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13. ABSTRACT *(Maximum 200 words)*

This project examines mechanisms by which human prostate carcinoma (PCA) cells undergo metastasis in an athymic nude mouse (male) model system since little attention has been devoted to these events for PCA. This includes PCA CWR22, CWR22R, and CWR21 xenografts adapted to tissue culture. To track tumor cells at the single-cell level and quantitatively, histochemical marker genes will be transfected for resolution as blue-, red-, or black-staining cells. Tissue culture lines of CWR21 have now been isolated for the first time--CWR21A and B; these are highly metastatic to several organ systems. Specific aim I examines the organ specificity of metastatic spread (particularly to bone and liver which escape detection in most animal models). LacZ-tagged CWR22R cells have been isolated and s.c. injected. We routinely observe micrometastasis to lung, liver, and bone (in select cases to brain). Specific aim II will test any significance for androgen-dependence or independence in metastatic spread, particularly for organ specificity. Androgen-relatedness of metastasis of CWR21A, B and lacZ-CWR22R cells is being evaluated. Specific aim III will evaluate possible interclonal cooperativity by injecting two different PCA cell types tagged with different marker genes; PAP-tagged CWR21A,B and CWR22.

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Lloyd A. Culp - 6/15/99

PI - Signature Date

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(1) Reprint: Culp, L.A., Lin, W.-c., Kleinman, N.R., Campero, N.M., Miller, C.J., and Holleran, J.L. Tumor Progression, micrometastasis, and genetic instability tracked with histochemical marker genes. Progress Histochemistry and Cytochemistry, **33**, No.3-4, 329-350 (1998).

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Introduction

Prostate carcinoma (PCA) in humans is most devastating because of its metastatic capability, particularly to lung, bone, and liver. Unfortunately, little experimental study has been dedicated to the mechanisms by which PCA undergoes its progression from the primary tumor in the prostate gland into these and possibly other organ sites. This project will examine these mechanisms more carefully by inserting a genetic tag into PCA cell types that light these cells up as specific colors (thereby readily distinguishing them under the microscope as blue, red, or black cells in a background of colorless tissue). These genetic tagging experiments with marker genes are powerful by permitting us to detect single tumor cells in virtually any organ and for quantitating micrometastasis to any target organ. By these means, androgen-dependent PCA cells will be tagged with one marker gene and androgen-independent PCA cells with another marker gene. Metastatic capabilities will be compared individually and when two cell types are mixed. In addition, metastatic-competent cells will be compared with nonmetastatic cells individually and when mixed to determine if metastatic cells can convey some metastatic potential to the other cell type. These experiments will be conducted in athymic mice which lack cell-mediated immunity and to verify the accuracy of this animal model system to the human disease process. They will also include injecting the PCA tumor cells into different sites of the animal to determine which site leads to the most accurate model of human disease. Overall, these studies will shed considerable light on the mechanisms by which prostate carcinoma develops the capacity to progress from the primary tumor into multiple target organs during metastasis.

Body

With regard to the original "Statement of Work" submitted with this application, a number of accomplishments have been or are being achieved in line with these objectives. These are as follows.

First, we have isolated two tissue culture cell lines from the CWR21 xenograft (human PCA) provided to us by Dr. Thomas Pretlow and described in the literature (3-6). These cell lines are referred to as CWR21A and B, are the first cell lines to be generated from this xenograft, and are being characterized by several approaches. (a) Dr. James Mohler (Univ. of North Carolina) is characterizing the androgen receptor gene in these cell lines to determine if it is wild-type or harbors the mutation characteristic of certain xenografts generated in the Pretlow laboratory. (b) We have provided these cells to Dr. Stuart Schwartz of this institution to perform cytogenetic analyses on these cells to verify the origin of these populations; this includes the average number of chromosomes per cell, the translocation of specific chromosome segments, deletion of specific chromosomes, and analyses of several chromosomal markers of the Pretlow xenografts. (c) These two cell lines have been injected into the subcutis of athymic nude mice by our laboratory to test tumorigenicity and possible metastasis. Both lines are incredibly tumorigenic, generating large primary tumors with 2-3 weeks, even without Matrigel co-injection; our

experience with other PCA cell lines indicates that 10-15 weeks is required to generate such large tumors. Also, CWR21A and B cell lines generate large lung metastases in all animals bearing primary tumors and metastases to other organs as well. They are probably the most metastatic PCA line that either our laboratory or the Pretlow laboratory has ever seen. In all these regards, these may be very important cell models of highly-metastatic PCA in the nude mouse model system. *Under "Statement of Work", these experiments address Aim 1b.*

Second, the CWR21A and B cell lines described above are now being transfected with the human placental alkaline phosphatase marker gene (PAP) to provide a histochemical marker that stains these cells differently from the *lacZ* marker gene in CWR22R cells (see below). These protocols are described in references 7-9. Transient transfections have optimized the transfection conditions using lipofectamine as the introducing agent. *Under "Statement of Work", these experiments address Aim 1a.*

Third, to test for androgen-dependence or independence of tumor formation and metastasis, CWR21A and B cells are being injected into mice with or without testosterone pellets being implanted into these animals (since young nude male mice have very low levels of endogeneous testosterone). In addition, castrated mice are also being tested for their responsiveness to these cells (with or without testosterone pellets). These experiments have just been undertaken and will test whether testosterone modulates the rapid rate of primary tumorigenesis and/or the metastatic behavior of these cells. *Under "Statement of Work", these experiments address Aims IIb and c.*

Fourth, the *lacZ* marker gene has been successfully transfected into CWR22R cells grown in culture, using our previously-described methods (7-9). Several stable transfectants have been isolated and are being characterized. These cells are referred to as LZ-CWR22R clone H, clone D, etc. These initial experiments are described in references (1) and (2), appended to this Progress Report. In brief, a wide spectrum of genetic and epigenetic stability of *lacZ* expression has been observed. Clone H is extremely stable with >80% of the cells staining blue with X-gal after 25 passages in culture. In contrast, clone D is very unstable with <15% of the cells staining after 7 passages and by 10 passages only a few cells stain. Clone B is intermediate-- by 10 passages, approx. 50% of the cells stain. The turn-off in expression of this marker gene is apparently not under control of hypermethylation of its promoter since addition of aza-deoxycytidine to these cells (a methylation inhibitor) has no effect on its expression in any of the clones. *Under "Statement of Work", these experiments address Aim 1a.*

Fifth, LZ-CWR22R-clone H cells have been injected into the subcutis of nude mice. They form tumors slowly over a period of 8-10 weeks that stain very blue, indicating the stability of the histochemical marker gene in vivo. In addition, virtually all tumor-bearing animals demonstrate blue-staining micrometastases in the lung, some of which grow into overt metastases. Approximately, one-half these animals have micrometastases in the liver and the bone; a subset of the liver micrometastases grow into excellent blue-staining overt metastases. These latter results are very important because there

has been great difficulty in other PCA model systems for identifying consistent metastasis to liver and bone which are major sites of the human disease. Therefore, we have a much more reliable model of the human PCA disease with these cells. In addition, a few animals display micrometastases in the brain for the first time; the significance of this finding remains to be determined. Some of these results are reported in reference (2). *Under "Statement of Work", these experiments address Aims 1b and d and IIb.*

Sixth, LZ-CWR22R-clone H cells have been injected with or without Matrigel to determine if this exogenous extracellular matrix alters the tumorigenicity and/or metastatic behavior of these cells. It is too early to determine the outcome of this experiment. *Under "Statement of Work", these experiments address Aims 1d and IIc.*

Seventh, several primary tumors derived from CWR21A and B cells, as well as from LZ-CWR22R-clone H cells, have been isolated back into culture and cell lines established. These tumor lines will be particularly valuable for testing clonal dominance in Aim III of this project. In addition, several overt metastases in lung and liver are being isolated back into tissue culture to test for organ specificity of metastasis. *Under "Statement of Work", these experiments address issues in Aims IIIa,b, and c.*

It should be noted that many of the experiments of Aim III cannot be undertaken until we obtain a PAP-transfected population of CWR21 A or B cells, as well as PAP-transfected tumors cells isolated back into culture as described in item 7 above.

A notable failure of this project thus far has been our inability to obtain a PCA cell line from the CWR22 xenograft of Pretlow and his collaborators (4,5). We have tried several approaches but have not been successful. A number of new initiatives and selective environments will be used in further attempts to obtain this important cell line (which would complete the series of all three xenografts with their unique biological and tumorigenic properties).

Key research accomplishments

- Isolation and characterization of CWR21 A and B cell lines from the CWR21 xenograft
  - •Androgen receptor status in these cells
  - •Cytogenetic analyses of these cell lines
  - •Aggressive formation of primary tumors and metastases to multiple organs
  - •Transfection with the PAP histochemical marker gene
  - •Androgen-dependence or independence in formation of primary tumors and metastasis
- •*LacZ* transfection into tissue cultured CWR22R PCA cells
  - •Genetic stability of the marker gene in various stable transfectants
  - •Testing hypermethylation regulation of marker gene expression
- Tumorigenicity and metastatic competence of *lacZ* transfectants of CWR22R cells
  - •Kinetics of primary tumor formation and X-gal stainability
  - •Micrometastasis to lung, liver, bone, and brain
  - •Overt metastasis in lung and liver
  - •Tumorigenicity/metastasis with or without Matrigel co-injection
- Re-isolation into tissue culture of primary tumor cells from CWR21 A and B and LZ-CWR22R clone H tumors

Reportable outcomes

(1) Reprint: Culp, L.A., Lin, W.-c., Kleinman, N.R., Campero, N.M., Miller, C.J., and Holleran, J.L. Tumor Progression, micrometastasis, and genetic instability tracked with histochemical marker genes. Progress Histochemistry and Cytochemistry, **33**, No.3-4, 329-350 (1998).

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Isolation of CWR21A and B cells lines from the CWR21 xenograft of human PCA

Generation of tissue cultured cell lines from primary tumors and overt metastases of CWR21A and B, as well as LZ-CWR22R-clone H

Conclusions

These experiments have successfully generated new tissue culture human prostate carcinoma cell lines from previously-characterized xenograft models of the disease in athymic nude mice. Furthermore, these cell lines are very aggressive in forming tumors and in metastasizing to multiple organs. The histochemical marker gene, *lacZ*, has been successfully transfected into CWR22R cells in tissue culture and the variable genetic stability of this gene determined. These cells generate excellent blue-staining primary tumors,

micrometastases to multiple organs (including lung, liver, bone, and brain), and overt metastases in some organs. Several primary tumors have been re-isolated back into culture to provide highly-selected subsets for future experiments to test organ specificity of metastasis and clonal dominance during metastasis. Overall, considerable progress has been made to generate a more accurate model of the human disease in the nude mouse model system and the mechanisms of metastatic progression of the disease.

Genetically-tagged PCA tumor cells generated in this project provide an extremely sensitive system for tracking progression and metastasis. In addition, other PCA cell lines generated in this project will be highly valuable reagents for other investigators of this important disease in an animal model system.

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Tumor Progression, Micrometastasis, and Genetic Instability Tracked with Histochemical Marker Genes

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With 6 Figures and 1 Table

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Contents

Abbreviations

ADH	*Drosophila* alcohol dehydrogenase gene
APSI	alkaline phosphatase gene-transfected, c-*sis*-transformed Balb/c 3T3 cells
AZA	2-aza 5′-deoxycytidine
CWR21, CWR22, CWR22R	three different xenografts of human prostate carcinoma carried in athymic nude mice
FDG	fluorescein-digalactoside substrate for β-galactosidase
hCD44s	human CD44s gene used for transfection
HA	hyaluronan
IG4-B, IG4-D, IG4-H	independent clones of *lac*Z-transfected human prostate carcinoma CWR22R cells
LCM	laser-capture microdissection
LZEJ	*E. coli lacZ*-transfected, EJ-H-*ras*-transformed Balb/c 3T3 cells
NK	natural killer cells
PAP	human placental alkaline phophatase gene
PCA	prostate carcinoma
RSV LTR	Rous sarcoma virus long-terminal repeat promoter
RT-PCR	reverse transcriptase-mediated polymerase chain reaction
SD	standard deviation
X-gal	substrate for assaying histochemically bacterial β-galactosidase in cells

Abstract

Mouse fibrosarcoma (3T3 cells transfected with different oncogenes), human neuroblastoma, or human prostate carcinoma cells have been genetically-tagged with different histochemical marker genes (*E. coli lacZ*, placental alkaline phosphatase, or *Drosophila* alcohol dehydrogenase). Injection into athymic nude mice permits their tracking at all stages of primary tumor formation and micrometastasis to various organs at the single-cell level. Two different tumor classes, tagged with different marker genes, can be tracked together. Primary tumors display regional dominance of one tumor class with exclusion of other classes. During micrometastasis, tumor cells are detected binding to the endothelium of lung blood vessels, followed by establishment of multiple-cell micrometastases. Micrometastases in some organs are transient while in other organs there is differential expansion into overt metastases. Tagged tumors also reveal the timing of angiogenesis of developing primary tumors and overt metastases. In all three tumor systems, there are three classes of genetic stability of marker gene expression in clonal populations-high stability, intermediate stability, and high instability. Instability in marker gene expression in one tagged prostate carcinoma system does not depend on a hypermethylation mechanism, suggesting a genetic basis for loss of activity. Use of histochemical marker genes, combined with laser-capture microdissection and various PCR methods, can now be used to evaluate gene activities in single or multiple tumor cells in virtually any organ and primary tumor of the animal model system.

1 Introduction and background

1.1 Rationale for histochemical markers

The tracking of tumor cells during their many stages of progression and metastasis has been an elusive goal in animal model systems. One exception to this problem has been melanoma studies in which the black pigmentation produced in the cytoplasm of these cells facilitated their identity as small clusters of cells, although single tumor cells were virtually unidentifiable (FIDLER et al., 1978; HEPPNER and MILLER 1983; MILLER et al., 1987; FIDLER and ELLIS 1994). This handicap of single-cell tracking was overcome by developmental biologists who sought to track the lineage of single or select cells in the embryo during the complex processes of differention. SANES et al. (1986) used the bacterial *lacZ* gene to track embryonic cell pathways virtually at the single cell level in the developing embryo. This use of the histochemical marker gene was followed by many similar analyses in developmental systems.

The pioneering studies of SANES et al. (1986) prompted us to implement the "nontoxic" *E. coli lacZ* gene in our own tumor progression studies since we had considerable difficulty tracking progression and metastasis of fibrosarcoma or neuroblastoma systems at their earliest stages (RADINSKY et al., 1987; CULP and BARLETTA 1990). Histochemical marker genes, such as *lacZ*, were advantageous to us over fluorescing markers, such as luciferase or green-fluorescent protein, because we sought to distinguish tumor cells in the normal tissue architecture; fluorescence analyses would have made this impossible. For H-*ras* transformed Balb/c 3T3 cells (LZEJ cells) injected into athymic nude mice, LIN et al. (1990a, b) showed the ultrasensitive detection of *lacZ*-tagged tumor cells in virtually any organ, both during spontaneous metastasis from the subcutaneous site and after experimental metastasis via injection into the tail vein. These studies identified micrometastasis not only to the lung but also to the brain and the kidney for the first time (LIN et al. 1990a, b; LIN and CULP 1992a). Using methyacrylate-embedded serial sections of lung or other organs, they also showed that single tumor cells could be readily identified in any organ (LIN et al. 1990b; LIN and CULP 1992b). Facile identification of single tumor cells also applied to the earliest primary tumors developing at the subcutaneous site (O'CONNOR and CULP 1994).

These methods were then extended to analysis of human neuroblastoma in an athymic nude mouse model system. Primary tumor development at intradermal or subcutaneous sites could be readily tracked with *lacZ*-tagged Platt neuroblastoma cells (KLEINMAN et al. 1994). They revealed the tissue-restrictive growth pattern of tumors and the neovascularization of expanding tumors. Histochemical marker gene tracking also permitted development of an orthotopic model of neuroblastoma. Injection of neuroblastoma cells into the adrenal gland resulted in subsequent metastasis to target organs not observed with intradermal or subcutaneous tumors (FLICKINGER et al. 1994; JUDWARE et al. 1995).

1.2 Alternative marker genes for tagging multiple tumor cell classes

The use of the *lacZ* gene proved so effective in our tumor analysis that we sought to use other marker genes, lighting cells up with different colored products, so that two or more tumor cell classes could be tracked simultaneously. For these reasons, human placental alkaline phosphatase gene (PAP) was developed as a marker gene since (a) background alkaline phosphatase staining could be eliminated with a 60° heat step without affecting activity of the transfected gene enzyme, (b) different histochemical substrates could be used to generate reddish-brown, black, or other colors readily distinguished from the blue staining of the *lacZ* β-galactosidase enzyme, and (c) the enzyme intercalated into the intracellular membranes of cells leaving the histochemical product bound within the cytoplasm (LIN et al. 1992). In a similar vein, the *Drosophila* alcohol dehydrogenase gene (ADH) was also developed since its histochemical products could be readily distinguished from the other two. Both ADH and PAP enzyme activities are nontoxic to animals cells and provide nonselective activities with which to study progression and metastasis.

All three genes were inserted into two different sets of integrating plasmids-one set regulated by the RSV LTR promoter while the second set was regulated by the cytomegalovirus late-gene promoter, providing options in terms of promoter regulation in our tumor systems. *Sis*-transformed Balb/c 3T3 cells (tumorigenic but not metastatic from the subcutaneous site) were then transfected with the PAP gene to yield the APSI cell line (LIN et al. 1992, 1993). When LZEJ and APSI cells were mixed and the mixture then injected into tail veins of animals, individual micrometastases could be identified and enumerated containing only one of the cell types. Moreover, a significant fraction contained both cell types, an indication of the multi-cell nature of the earliest experimental micrometastases. These studies also demonstrated that the high-metastatic LZEJ cells could facilitate the metastatic competence of the normally-nonmetastatic APSI cells (LIN et al. 1992, 1993).

1.3 Topology of two tumor cell classes as primary tumors develop

Development of the two-marker-gene system (LIN et al. 1992) permitted us to examine the development of primary tumors when mixtures of LZEJ and APSI cells were injected subcutaneously (LIN et al. 1993). We might have expected that both tumorigenic cell classes would be completely intermixed in the primary tumor. As shown in Fig. 1, a surprising finding emanated from these studies. Staining yielded a pattern of homogeneously-staining regions of each tumor class in an overall multi-regional pattern (Fig. 1). Each cell class was dominant in specific regions of the primary tumor with only limited intermixing of the two cell classes at their interfaces. This was the case in more than 20 primary tumors examined, indicating that this regional growth pattern of each tumor cell class was the general rule for these two very related classes (the difference being whether the human EJ-*ras* or c-*sis* oncogenes were the transforming principal).

Fig. 1. Topology of two tumor cell classes in a primary tumor. LZEJ (*lacZ*-transfected, H-*ras*-transformed 3T3) and APSI (PAP-transfected, c- *sis*-transformed 3T3) were suspended from separate tissue cultures, mixed in suspension, and then injected into the subcutis of an athymic nude mouse (Lin et al. 1992, 1993). When a 5 mm-diameter tumor had developed, the animal was euthanized and the tumor excised. The intact tumor was then fixed with a formaldehyde/glutaraldehyde solution and then sequentially stained-first, for PAP enzyme activity and then for β-galactosidase activity. APSI cells stain reddish-brown while LZEJ cells stain blue. It is apparent that the two tumor cell classes concentrate into regions of homogeneity in the primary tumor and are not completely intermixed.

Two possibilities for the regional dominance in these primary tumors come to mind. First, each cell class may migrate in the earliest primary tumor to form homogeneous aggregates in each region. Thus, each aggregate would give rise to a relatively homogeneous population of only LZEJ or APSI cells. Alternatively, there may be clonal dominance of one cell type over another based on competitive advantage of the tissue architecture in that location. In others words, LZEJ cells would outgrow APSI cells in some regions because they have an extracellular matrix or growth factor advantage while the reverse would be true in other regions. Clearly, this is an important finding since it indicates that tumor cells are highly discriminatory in their growth patterns. It also offers a model system as to how minor subpopulations could develop in specific regions of a large primary tumor.

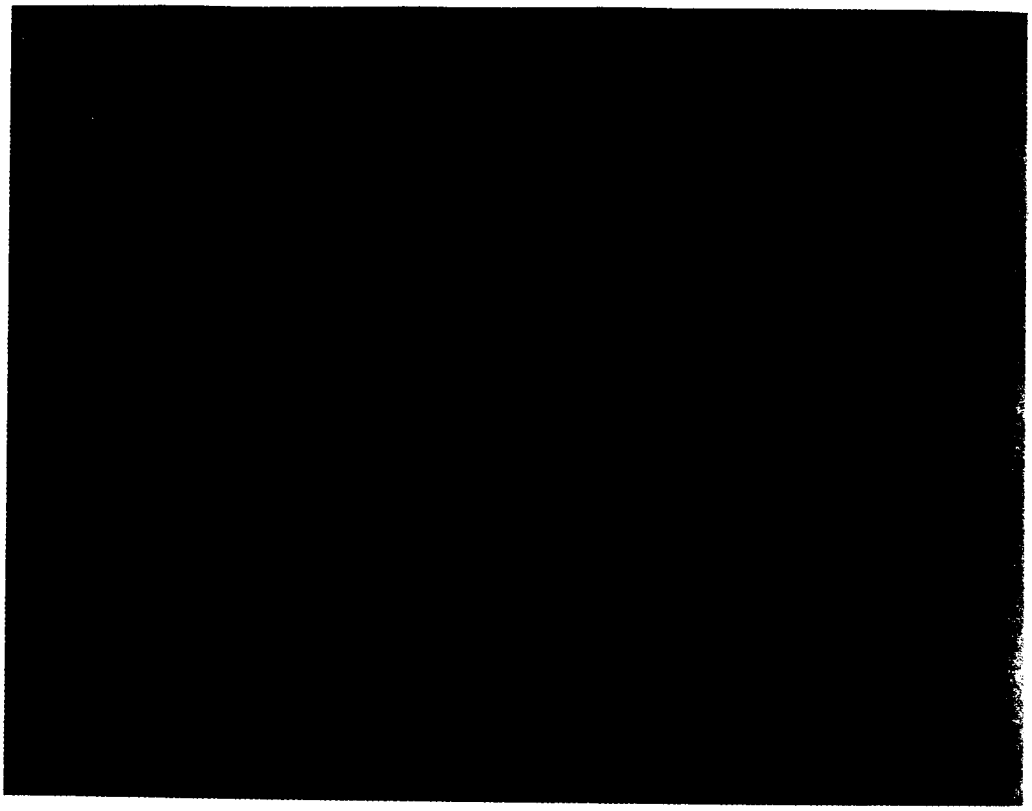


Fig. 2. Tumor cells adhering to the endothelium of a blood vessel. *Ras*-transformed, *lacZ*-tagged Balb/c 3T3 cells (LZEJ) (LIN et al. 1990a, b) were injected into the tail veins of athymic nude mice. At 30 minutes post-injection, animals were sacrificed; the lungs were then excised, fixed, and embedded in methacrylate at −20 °C and cut into 4 μm sections for X-gal staining (LIN et al. 1990b). Blood vessels were detected with alkaline phosphatase staining (red-staining; broad solid arrow) while tumor cells stained blue with X-gal (small, open arrows). Note that there are tumor cells adhering to the endothelium within the blood vessel, possibly in the process of extravasation, while other tumor cells have already escaped into the tissue space. × 400.

2 Micrometastasis development

2.1 Tumor cell binding to endothelium of lung blood vessels

A major motivation for using histochemical marker genes was the ultrasensitivity for detecting micrometastases at their earliest time points. Figure 2 shows the power of this methodology. LZEJ cells were injected into the tail veins of nude mice and then animals sacrificed at various times from 5 minutes to >24 hours later. At the very early time points (e.g., 30 minutes in Fig. 2), serial sectioning of the lungs revealed some tumor cells

which had escaped from blood vessels into tissue sites and, in some exceptional cases as shown, tumor cells could be shown binding to the endothelium of the small blood vessels of the lung.

This ability to detect single tumor cells at the endothelium will permit us to ask some very specific questions about gene regulation at the earliest time points of micrometastasis initiation. As an example, laser-capture microdissection has recently been developed to permit investigators to evaluate, by RT-PCR, gene activities in single tumor cells in fixed sections of tissues (EMMERT-BUCK et al. 1996). Histochemical tagging of tumor cells permits us to carefully identify their location and number. In combination with laser-capture microdissection, a vast array of very important questions can now be addressed directly with *in vivo* analyses (see section 5.2 for expansion of this issue).

2.2 Multiple tumor-cell foci – possible mechanisms of formation

In our early studies of micrometastasis to the lung using LZEJ cells (LIN et al. 1990a, b, 1992, 1993), it became clear that single tumor cells were not populating the blood vessels of the lung but rather collections of seveal tumor cells, frequently 2–6 at each locus. Serial sections of one of these micrometastases are shown in Fig. 3 where 6 cells can be identified in one focus. Furthermore, the clearing of approximately 97–8% of micrometastases within 24 hours was an all-or-none phenomenon – i. e., all cells of these multiple-cell foci were cleared rather than the average number of cells per focus decreasing from 5 or 6 down to 1 or 2 cells. This indicated that NK cells were completely competent for clearing all cells in a multiple-cell focus, not just a few cells. While the micrometastasis shown in Fig. 3 is captured at the 1 hour time point, virtually the same results would be obtained at the 24 hour time point when "clearing" of selected micrometastases had been completed.

These results raise the intriguing issue as to what makes the persistent micrometastases resistant to the animal's clearance mechanisms. This question was first raised by the studies of Fidler, Nicolson, and their colleagues and remains unanswered today (FIDLER et al. 1978; NICOLSON 1993; FIDLER and ELLIS 1994). There are at least three different mechanisms that would explain the persistence and stabilization of the few percent of all early micrometastases. First, these micrometastases may colonize the lung at special sites that are not amenable to NK cell or other immune effector cell action. Second, these persistent micrometastases may penetrate the endothelium most effectively and thereby evade NK cell action. Finally, the cell surface properties of this select subset of tumor cells may be different from the majority and somehow make them tolerant of any killing mechanism mediated by NK and other immune effector cells. Again, this is an area of tumor biology that is clearly understudied, requiring greater molecular and mechanistic analysis.

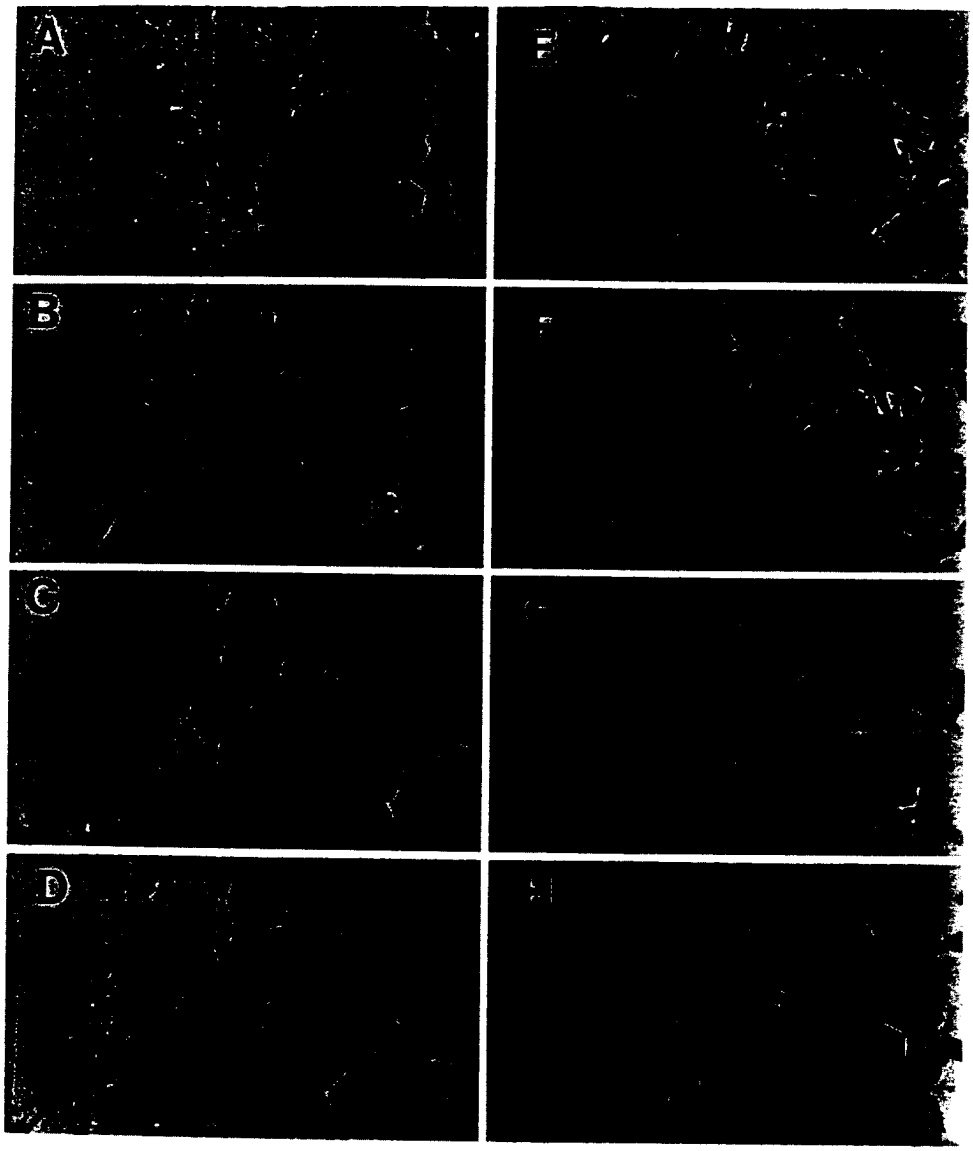


Fig. 3. Serial sections of a single micrometastasis. LZEJ tumor cells were injected into the tail vein of an athymic nude mouse. At 1 hour postinjection, the mouse was sacrificed; the lungs were excised, fixed, and X-gal-stained. One micrometastasis was carefully cut out of the tissue which was serially sectioned to give the sections [A]–[H] shown here. Note that this micrometastasis is comprised of 6 or 7 cells (the broad solid arrows). The bent arrows indicate a tangential turning point of this micrometastasis along a lung air sac structure. × 360. (Taken from LIN and CULP 1992b, with permission.)

2.3 Differential expansion into overt metastases

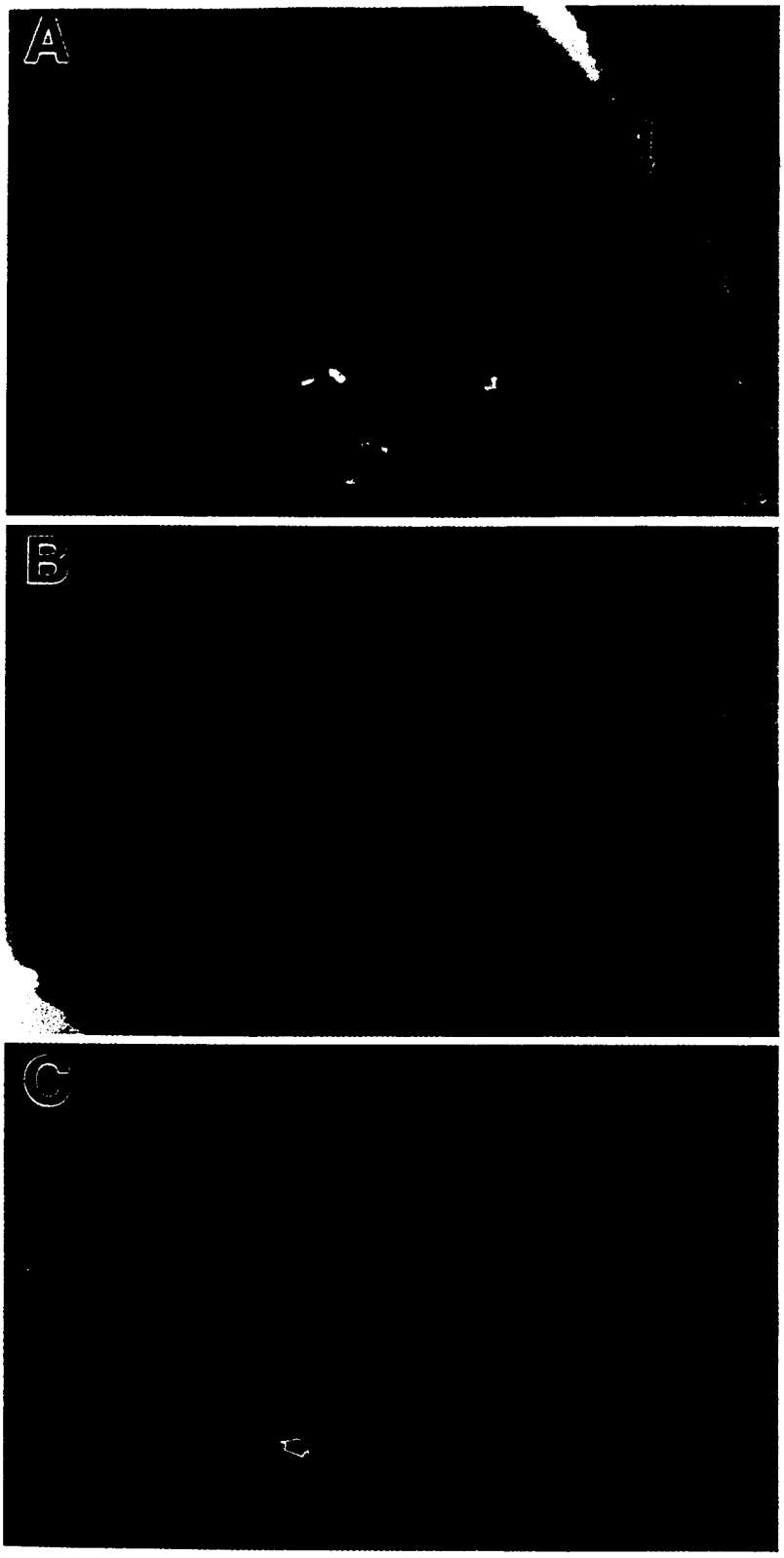
The questions raised above led us to investigate the fate of micrometastases in the lung and other organs. In the brain, micrometastases of LZEJ survived for 1–3 weeks and then competely disappeared, possibly because they encountered an environment hostile to their survival (LIN and CULP 1992a). Such environmental factors may include an inappropriate extracellular matrix with which to interact and/or inadequate supplies of growth factors because of the blood:brain barrier.

In contrast, the fates of micrometastases in the lung were far more complex (LIN et al. 1992, 1993). When APSI cells were injected alone into tail veins, they generated micrometastases in the lung that rarely developed into overt metastases. In most cases, these micrometastases disappeared with time. In contrast, when LZEJ and APSI cells were co-injected, micrometastases of both tumor classes persisted and many more APSI foci developed into overt metastases, as did LZEJ foci as expected. This suggested that the LZEJ cells potentiated outgrowth of APSI micrometastases in this "foreign" tissue.

When LZEJ cells were injected alone into tail veins, the fates of micrometastases in the lung were varied over a 2–4 week period (LIN et al. 1990b, 1993). Some micrometastases failed to expand into overt metastases while others did expand. Whether micrometastasis expansion occurs or not is an important issue in mechanistic studies of these events. Using nonfixed lungs from euthanized animals and FDG substrate for *lacZ*-coded β-galactosidase (fluorescein-digalactoside which penetrates living cells without killing them and generates a fluorescing product with the bacterial enzyme), it will be possible to identify "live" micrometastases and distinguish them from overt metastases. These two cell classes can then be recovered from lungs, grown out in culture, analyzed for their gene regulation patterns, and reinjected into a second group of animals to determine if these patterns breed true.

2.4 Earliest detection of angiogenesis as primary tumors develop

During analyses of the development of primary tumorigenesis using *lacZ*-tagged human neuroblastoma, KLEINMAN et al. (1994) raised the question as to when blood vessels could be detected near or within the developing tumor. To do so, they used cardiac perfusion of fixative to stably fix the red blood cells within blood vessels, making their visualization much more facile. It then became clear that the smallest blood vessels could be seen sprouting toward the tumor within 48 hours. An example is shown in Fig. 4. Within several days one or more of these very small vessels were developing into much larger vessels that eventually became quite large. This important finding indicates that the tumor cells that become established in the subcutis within 24 hours (O'CONNOR and CULP 1994; KLEINMAN et al. 1994) contain information for inducing angiogenesis. These angiogenic events have become central to studies of tumor progression and involve a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large array of negative and positive chemokine factors (BLOOD and ZETTER 1990; FOLKMAN 1995). Using histochemically-tagged tumor cells, it will now be possible to modulate the activities of specific genes in these cells while in culture and then assay their relative ability or inability to promote blood vessel sprouting at virtually any injection site in the host animal.

3 Human prostate carcinoma cell "tagging" with marker genes

3.1 Selection of cell systems

Progression and metastasis of human prostate carcinoma (PCA) has been a very understudied area of tumor biology, principally because of the paucity of excellent cell systems in culture and suitable animal models (PRETLOW et al. 1994; LALANI et al. 1997). The most commonly used cultured cell systems include PC3, DU145, and LNCaP; however, these were isolated from metastases in human patients and therefore represent highly selected subsets of tumor cells. Greater hope was generated for more and better cultured cell systems with the development of many xenografts of human PCA primary tumors in nude mice by the Pretlows and their collaborators (WAINSTEIN et al. 1994; CHENG et al. 1996; NAGABHUSHAN et al. 1996). They took advantage of the stabilization of transplant of human primary tumor tissue into nude mice co-injected with Matrigel. Three promising cell systems have now evolved from these studies, including CWR21 (androgen-dependent, metastatic), CWR22 (androgen-dependent, nonmetastatic), and a relapsed variant of the latter (CWR22R-androgen-independent, metastatic to lungs of nude mice). CWR22R xenografts have now been adapted to growth in tissue culture by Dr. James Jacobberger and his colleagues at Case Western Reserve University (manuscript submitted for publication) and offer an excellent population of primary PCA tumor cells for study.

Fig. 4. Angiogenesis during primary tumor establishment. *LacZ*-transfected human neuroblastoma tumor cells were injected into the dermis or into the subcutis between two India ink spots on the skin. At the indicated times, animals were sacrificed and fixative solution perfused into the left ventricle of the heart to preserve blood vessel red staining and contrast. The injection sites were then excised, fixed, and stained with X-gal.
A: Tumor cells 48 hours after injection into the dermis. Very small blood vessels are beginning to branch toward the tumor (small arrows) from a major blood vessel (large arrow). × 87.
B: A tumor 2 weeks after dermal injection. A large number of small blood vessels are invading the tumor site (small arrows) and in select cases expanding into a major blood vessel (large arrow). × 87.
C: A tumor 3 weeks after injection into the subcutis. Much of this tumor has lost X-gal-stainability with the exception of one region (open arrow). The tumor is now fed by a major blood vessel (large arrow), many intermediate-sized vessels (small arrow), and very small micro-vessels (black arrowhead). (Taken from KLEINMAN et al. 1994, with permission).

3.2 Major issues to address

Our laboratory has successfully transfected the *lacZ* gene into CWR22R human PCA cells (CAMPERO, HOLLERAN, MILLER, and CULP, unpublished data) and is undertaking a variety of studies with these tagged cells. Of particular note, virtually all experimental human PCA tumor model systems have failed to identify metastasis to the bone and liver which are common sites for the human disease. Metastasis to the lung in these animal models is much more common (PRETLOW et al. 1994; LALANI et al. 1997). *LacZ*-tagged cells should facilitate the detection of tumor cells in liver and bone (see below on this point) and provide opportunity for evaluating the relative significance or insignificance of androgen ablation or supplementation in these processes. Of equal importance is a comparison of ectopic or orthotopic injection sites, since the latter may provide a more accurate pattern of metastatic spread to all three organs (STEPHENSON et al. 1992; FU et al. 1992; SATO et al. 1997).

With regard to acquisition of metastatic competence, we recently reported the plasticity of expression of CD44s during progression and metastasis in the mouse fibrosarcoma system (KOGERMAN et al. 1997a, b, 1998). This isoform of CD44 is the simplist form, is exclusively expressed in fibroblasts, and binds hyaluronan (HA) when "activated" at the cell surface by one of several possible mechanisms (LESLEY et al. 1993; CULP and KOGERMAN 1998). Transfection of an overexpressing human CD44s gene into nonmetastatic *sis*-transformed 3T3 cells converted them into highly metastatic cells (KOGERMAN et al. 1997a). Micrometastatic tumor cells, reisolated from lungs, expressed very high levels of the human antigen while large primary tumors or overt metastases had lost expression of the human gene, demonstrating the plasticity of expression of the transfected human gene in this system. Down-regulation of hCD44s in the large tumors occurred by hypermethylation of its promoter. Furthermore, transfection of the human gene into 3T3 cells made them tumorigenic *and* metastatic (KOGERMAN et al. 1998). These results indicate that the HA-binding and overexpressed human protein (mouse CD44s in these cells does not bind HA) conveyed metastatic competence but was antagonistic for outgrowth of tumors, whether they be primary or secondary.

An experimental metastasis model was then used to explore the mechanism(s) of this conveyance (KOGERMAN et al. 1997b). hCD44s-overexpressing cells colonized and stabilized micrometastases in the lungs 7–10-fold over nonexpressing cells. Furthermore, mixing overexpressing/transfected cells with nonexpressing cells prior to injection into tail veins revealed that the nonexpressing cells do not compete with the overexpressing cells for their more efficient colonization of the lung. These differences were also maintained when comparing primary tumor cell populations that had lost hCD44s overexpression with lung micrometastatic tumor cells that conserved overexpression; the micrometastatic cells were severalfold more effective at stably colonizing the lung. Therefore, the HA-binding and overexpressed hCD44s protein must provide a more effective mechanism of colonizing the lung at the endothelium and/or promote more

effective stabilization of each micrometastasis. It is possible that hCD44s at the tumor cell surface binds to HA on the surface of the endothelium, conveying this competence. In this latter regard, it will be critical to test HA-nonbinding mutants of hCD44s in this system (KOGERMAN et al. 1997b). Clearly, the use of histochemically-tagged cell systems can facilitate these mechanistic studies. We can also transfect an overexpressing CD44 gene into *lacZ*-tagged CWR22R prostate carcinoma cells and evaluate this protein's ability to modulate progression and metastasis of this particular tumor type.

3.3 Bone micrometastasis and overt metastasis

Neuroblastoma tumor cells injected into the subcutis or the dermis failed to metastasize effectively to any organ, even though some of these human neuroblastoma isolates were from metastases in the patient (KLEINMAN et al. 1994; FLICKINGER et al. 1994; JUDWARE et al. 1995). This indicated the difficulty of paralleling the human disease process in an animal model. In contrast, orthotopic injection of these same tumor cells into the adrenal gland produced metastases to the lungs and several other organs of nude mice (FLICKINGER et al. 1994; JUDWARE et al. 1995).

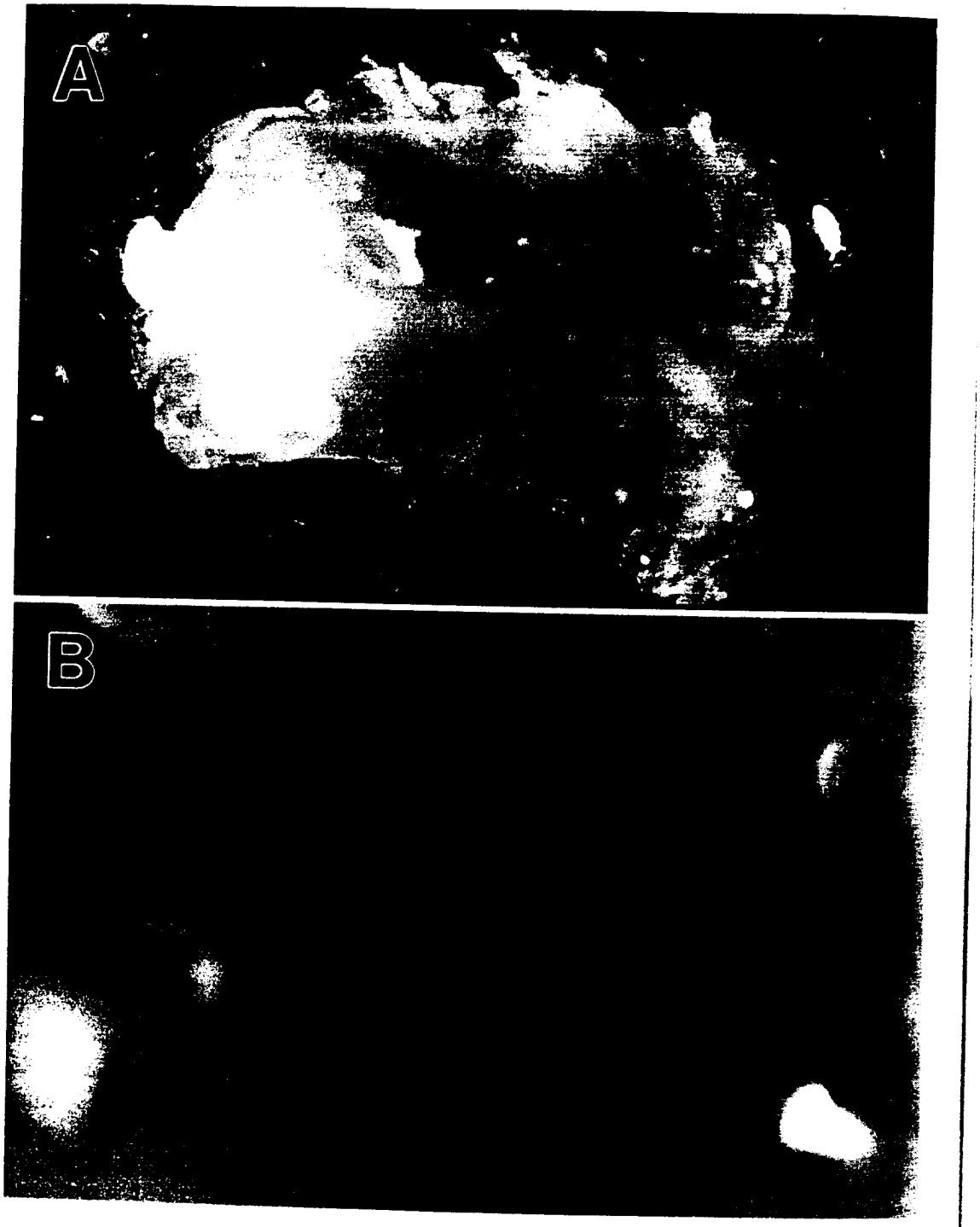
A notable target organ for neuroblastoma metastasis is bone, an organ which is rarely considered or identified as a target in animal models. As shown in Fig. 5, bone metastasis could be readily detected using *lacZ*-tagged neuroblastoma cells after adrenal gland injection while a control mouse not injected with tumor cells failed to yield any X-gal background staining of its vertebrae as expected. These micrometastases occurred at the surface of the bone and in some cases grew out as significant nodules of cancer in this site. These findings raise confidence that bone metastasis using prostate carcinoma models may eventually identify routing of *lacZ*-tagged PCA cells to this important organ in the animal model.

4 Genetic instability in tumor populations deciphered with marker genes

4.1 Mouse fibrosarcoma studies

Histochemical marker genes such as *lacZ*, PAP, and ADH offer no particular selective advantage to cells expressing them. Therefore, they offer an ideal opportunity to evaluate genetic stability or instability of their expression in many independent transfectants of tumor populations (LIN and CULP 1992a). A full spectrum of genetic instabilities have now been documented in our three tumor systems.

Using oncogene-transformed mouse 3T3 cells, different degrees of expression stability were observed with LZEJ or APSI cells (LIN et al. 1992; 1993). LZEJ cells persisted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in their high levels of *lacZ* expression whether grown in culture for 10–15 passages in the absence of selection drug or whether developed into large tumors in nude mice. In contrast, large tumors of APSI frequently had segments that failed to stain for the PAP marker gene, as did cells grown in culture for > 5 passages. Therefore, APSI tumor cells were rapidly losing expressability of the PAP gene for any one of a number of reasons. One possibility is that hypermethylation occurred at the PAP promoter, leading to loss of activity. This was observed for human CD44s gene in our transfected *sis*-transformed cells reported above (KOGERMAN et al. 1997a, 1998). Altered methylation appears to be a much more common pattern of changing gene expression in tumor cells than was originally thought. A second mechanism is a DNA rearrangement in APSI cells leading to truncation, deletion, or interrupted promotion of the PAP gene. A third possibility is loss of the chromosome bearing the integrated PAP gene since most transfectants carry only one copy of the histochemical marker gene. Greater study should be dedicated to the use of histochemical marker genes as tools, to decipher how tumor cells generate their genetic instability, particularly since this instability is essential for generating metastatic variants.

4.2 Human neuroblastoma studies

The same variety of marker gene stability of expression was observed in human neuroblastoma cells tagged with *lacZ* (KLEINMAN et al. 1994). One clone was particularly stable, generating well-staining cells after many passages in culture or growth *in vivo* into very large tumors. A second transfectant displayed an intermediate stability and a third clone was highly unstable, rapidly losing stainability as very small primary tumors.

4.3 Human prostate carcinoma studies

The recent isolation of *lacZ*-transfected CWR22R human prostate carcinoma cells (CAMPERO, HOLLERAN, MILLER, and CULP, unpublished date) has provided opportu-

Fig. 5. Metastasis to bone detected with *lacZ* staining. *LacZ*-transfected human neuroblastoma tumor cells were injected into the adrenal gland, an orthotopic site for this tumor (FLICKINGER et al. 1994; JUDWARE et al. 1995). Animals were euthanized at various time points, various bone structures excised from the animal, and these bones fixed and stained with X-gal to detect micrometastases.
A: A spinal column vertebra from an animal that has not been injected with tumor cells. Note the complete absence of X-gal staining of the bone.
B: X-gal-stainable micrometastases observed in spinal vertebrae from an animal 55 days after adrenal injection. Note the multiplicity of micrometastases in the vertebrae by this time point.

nity to analyze the relative genetic stability of multiple independent clones of PCA. As shown in Fig. 6, X-gal staining of late passage cells, grown in the absence of a selection drug for > 10 passages, demonstrates diversity in stability. Clone IG4-H is very stable in its expression over this lengthy period of time. Clone IG4-B has an intermediate level of stability while clone IG4-D is so unstable that virtually no blue-staining cells could be observed after 10 passages in culture.

These stability comparisons were quantitated by dilution plating of these cells into large tissue culture dishes, their outgrowth as individual colonies, and then enumeration of the percentage of blue-staining colonies (Table 1). Clone IG4-H, after ten passages in culture in the absence of a selecting drug, contains almost 90% of its population as *lacZ*-expressing cells. This confirms a remarkable stability of expression of this particular tumor subpopulation. In contrast, clone IG4-B has lost much of its activity and is reduced to only 27–35% colony staining. Clone IG4-D has lost all stainability. To test whether some of this loss of expression could be due to hypermethylation of the LTR promoter regulating *lacZ* in these transfectant cells (KOGERMAN et al. 1997a), colonies were also grown in the presence of the methylation inhibitor, 2-aza-5'-deoxycytidine to test revertability of expression. As shown also in Table 1, the azadeoxycytidine did not significantly increase the percentage of staining colonies in clones IG4-B or IG4-D (this would not have been expected in the excellent-staining clone H cells). Therefore, promoter hypermethylation is probably not the mechanism of down-regulation of *lacZ* expression in these unstable clones. Colonies were then grown in medium containing the selection drug G418 to test for plasmid persistence. This did not increase the percentage of blue-staining colonies in either clones IG4-B or D (again, no change was expected in the excellent-staining clone H cells) [data not shown]. Other genetic or epigenetic mechanisms must be responsible for this loss of *lacZ* gene activity. These processes can now be compared with those of our fibrosarcoma and neuroblastoma populations to resolve any tumor-specific or commonly-observed mechanisms of gene activity loss.

Fig. 6. Genetic stability of *lacZ* in prostate carcinoma cells. Human prostate carcinoma CWR22R tumor cells, transfected with *lacZ*, were isolated as three separate transfectant clones (IG4-clone B, D, and H). These three clones were then grown in culture for > 10 passages without any selection drug (G418) to test the relative stability of X-gal-stainability of these three clones. Colony analyses and quantitation of *lacZ* stability is provided in Table 1. In this figure, mass cultures of the three clones were stained in the absence of any selection drug.
A: Clone D-note the virtual absence of any staining, except for an isolated single cell (arrow).
B: Clone B-approx – one quarter of these cells have retained *lacZ* expression while the remainder have lost expressability.
C: Clone H-virtually all of these cells have retained stainability, indicating remarkable stability of *lacZ* expression in the absence of any selection pressure to maintain its expression.

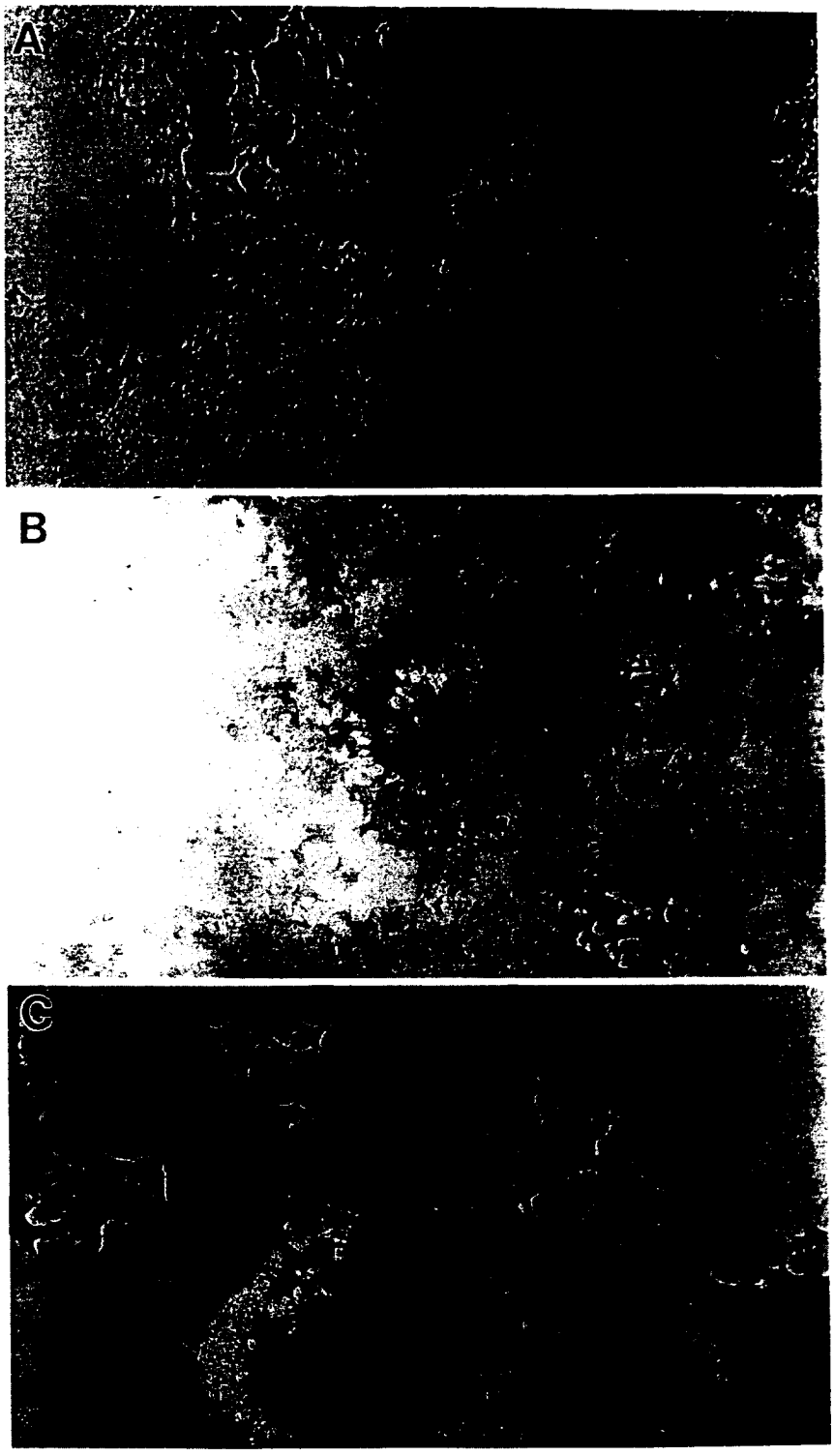
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Table 1. Genetic stability of *lacZ* marker gene expression in prostate carcinoma cells.

	Clones[a]	
	IG4-B	IG4-H
Colonies/plate[b] ± (SD)		
– Aza[d]	198.0 colonies ± 35.9	170.3 colonies ± 53.2
+ Aza[e]	203.3 colonies ± 46.6	206.7 colonies ± 67.7
% Blue colonies[c] ± (SD)		
– Aza	27.2% ± 10.4	90.3% ± 1.77
+ Aza	34.5% ± 12.6	87.9% ± 10.9

[a] The prostate carcinoma cell line CWR22R was transfected with the plasmid pRSVlacZII. This plasmid carries bacterial *lacZ* under the control of the RSV LTR promoter as well as a neomycin^R gene. Independent transformation events produced several stably-transformed cell lines, such as IG4-B, IG4-H, and IG4-D, which originally expressed *lacZ* in nearly all cells. Cells used in this study had been grown for 10 passages in the absence of selective media to examine whether known genetic instabilities of carcinoma cells might affect expression of the *lacZ* marker gene. Approximately 5000 cells were plated onto each of twelve 100 mm tissue culture plates and colonies were grown at 37 °C. After 7 or 10 days (lines B and H, respectively), half of the plates were supplemented with 3 μM 2-aza-5'-deoxycytidine. Five days later cells were fixed and stained. Virtually all IG4-D colonies were nonstaining after lengthy growth in nonselective medium, indicating considerable instability of *lacZ* in this particular clone.

[b] There was no significant difference in the number of colonies per plate between clones IG4-B and IG4-H ($p = 0.569$, ANOVA) or among plates with and without 2-aza-5'-deoxycytidine ($p = 0.326$, ANOVA).

[c] There was no significant difference in the percentage of blue colonies between 2-aza-5'-deoxycytidine plus and minus plates ($p = 0.887$, ANOVA). However, IG4-H had a significantly higher percentage of blue colonies than IG4-B ($p < .0001$, ANOVA). A third clone, IG4-D, showed negligible x-gal staining with or without 2-aza-5'-deoxycytidine. Therefore, data from this clone was not included in the table.

[d] – Aza = colonies grown in the absence of 2-aza-5'-deoxycytidine.

[e] + Aza = colonies grown in the presence of 3 μM 2-aza-5'-deoxycytidine.

5 Gene regulation in single tumor cells – importance of *in vivo* analyses

5.1 Two distinct functions for marker genes in vivo – identification and gene instability studies

The studies described above demonstrate how histochemical marker genes can be used in two very different ways. Initially they have been used to locate very small primary tumors and micrometastases at virtually any organ in order to assess these very early

events. This includes quantitation of micrometastases in various target organs. In addition, the ease of identification and quantitation of histochemical marker genes offers ideal opportunity to quantitate and evaluate various mechanisms for genetic instability in select tumor subpopulations. Such information is critical for predicting how rapidly metastatic variants could be generated by this genetic instability. Marker genes can be used in these gene stability studies because they are "irrelevant" to the successful growth and survival of tumor cells – i. e., they do not offer any selective advantages or disadvantages. Combined with laser-capture microdissection (EMMERT-BUCK et al. 1996) and other high-resolution analyses of tumor populations *in vivo*, we can now directly evaluate genetic instability of tumor populations in various tissue sites in the experimental animal. This will allow us to determine whether orthotopic or ectopic sites differ in their conveyance of genetic instability to specific tumor populations. Angiogenesis at the primary tumor site may also be a significant factor in selecting rare tumor cell subsets once genetic instability has generated opportunistic variants in the population. Overall, there has been little study as to the significance of the *in vivo* environment for conveying how rapidly gene stability changes and which subsets competitively overgrow their neighbors.

5.2 Laser-capture microdissection in combination with marker gene studies

Laser-capture microdissection (LCM) is a powerful tool for evaluating gene regulation in single or a few tumor cells in any tissue of the animal (EMMERT-BUCK et al. 1996). Histochemical markers permit us to precisely identify the location of tumor cell subsets whose gene regulation mechanisms may be most important. This may be the subset proximal to small blood vessels during early angiogenesis. The subset proximal to host organ connective tissue may also be of considerable interest because of the speculated synergy between tumor cell gene regulation and factors provided by neighboring host cells. A very important subset to evaluate by LCM includes cells in the act of intravasating into blood vessels, the earliest stage of metastatic migration; section 2.1 demonstrates the feasibility of such a study. Finally, gene regulation in the tumor cell subsets that are extravasating into different organs of the same animal may indicate whether common mechanisms operate in these different organs or whether there are true organ-specific metastatic steps involved.

5.3 Chimeric genes to track functional expression of specific gene classes during progression and metastasis

The active sites for enzyme activities in histochemical marker genes are being reduced to much smaller sequences of protein. Minigenes containing these minimal sequences retaining histochemcal activity can now be fused with the N-terminal or C-terminal

domains of receptors and other molecules of tumor cells felt to be highly significant in metastatic progression. Examples would include CD44s and its variant isoforms, the extracellular matrix receptor integrin subunits, and receptors for growth factors. By transfecting chimeric genes into tumor cells in which the histochemical activity is actually fused to the receptor protein, investigators can localize the optimal functions of these receptors in various topological sites of primary tumors, micrometastases, and overt metastases to test plasticity of expression. This may also be a case where a fluorescent marker, such as luciferase or green fluorescent protein, provides greater resolution in tissue sections than the conventional histochemical markers described above.

An alternative approach can also be used here. A dominant-negative inhibitor of a tumor cell receptor can be fused with the histochemical marker. This chimeric gene, transfected into cells, may demonstrate where and how the dominant-negative regulator of receptor function might modulate tumor growth, progression, and/or metastatic spread.

The methodologies are now available for asking and answering some very important questions at the level of single tumor cells in virtually any tissue site. This also applies to specific gene activities and to their possible modulation during these early events. The next decade should generate some very exciting and important mechanistic information about tumorigenesis and metastasis.

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Subject index

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Targeting the Metastatic Process

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Running Title: Tracking tumor progression and micrometastasis

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(I) Recent Approaches to Dissect Tumor Progression and Metastasis in Animal Model Systems

Metastasis of a tumor to target organ sites is a very complex series of events (1-7). It must involve highly selected primary tumor subpopulations, blood and/or lymphatic vessels, and specific organs amenable to "receive" the tumor cells. At least six to eight different steps can be envisioned in this migratory sequence, each of which must require specific and specialized gene products to be successful. One of the most impressive aspects of cancer metastasis is the remarkable versatility of tumor cells to generate such sophisticated subpopulations.

Angiogenesis is one critical consideration during metastasis (1,10,11). Not only must metastatic tumor subsets intravasate and extravasate currently-established blood vessels, they must also secrete the critical factors required at their foreign target sites to induce new blood vessel formation if micrometastases are to grow successfully into overt metastases. Since other chapters in this volume deal with angiogenesis regulation specifically, no further comment will be made on the significance of these specialized gene products and their functions in this chapter.

There are analogies between the evolution of metastatic variants in the primary tumor and some complex events that occur during normal embryonic development (12,13). For example, development of the neural crest generates multiple cell subsets that migrate to other "foreign" regions of the embryo where they differentiate into adrenal gland cells, peripheral neurons at many sites,

melanocytes throughout the skin, and some facial bones. Developmental biologists have shown that these complex patterns require a shifting in the expression of many genes, not just one or a few genes (13).

Likewise, metastasis to various target organs from the site of the primary tumor must involve a large array of gene activities. This is evident since the primary tumor is embedded in its "native" cellular environment while all target metastatic sites must be viewed as "highly foreign" to that particular tumor cell (1-6). This complexity is compounded by intravasation/extravasation of blood vessels and/or lymphatic vessels during these events and by the fact that a particular class of primary tumor does not metastasize to only one target site (although one site might be highly preferred during early events) but to multiple sites.

Targeting multiple tissue sites presumably requires different, but overlapping, gene classes in tumor cell subsets. In support of this hypothesis, evidence is mounting for organ-specific regulation of tumor cell genes (7,8; see sections II and III below). Furthermore, these new patterns of gene expression in metastatic tumor subsets are a reflection of the genetic instability of cells in the primary tumor, permitting select subsets to be successful and efficient in the multiple steps involved. Therefore, it is highly unlikely that there is a master "metastasis-control" gene in any tumor type that oversees these complex events (9); rather, it is genetic instability leading to many subsets of tumor cells that guarantees success in metastasis to lung, liver, bone, bone marrow, brain, and other target organs.

Another consideration in these experimental paradigms of tumor progression and metastasis is the pattern(s) by which genes change their expression. It is highly likely that some genes are turned off to generate metastatic variants, while other gene classes are turned on to execute specialized functions. There is also evidence, reviewed in section III, of reversible expression of at least one gene during progression and metastasis, a concept predicted by Nicolson (4) many years ago.

Our studies, summarized in this review, involve three different tumor systems. The first is the mouse fibrosarcoma system in which Balb/c 3T3 cells are transfected with one of three different oncogenes-- the human EJ-H-*ras* oncogene, the mouse Ki-*ras* oncogene, or the human c-*sis* oncogene (14). The second system is human neuroblastoma, with or without N-*myc* oncogene amplification (15). More recently, we have initiated studies of the progression and metastasis of new human prostate carcinoma (PCA) tumor cell systems (16). These PCA studies are particularly significant since so little is known in the human disease and/or model systems regarding its mechanism(s) of metastatic progression. In all cases, in vivo studies have been analyzed in athymic nude mice and compared with phenotypes of respective tissue culture cell subpopulations.

These three tumor systems will be reviewed from three different methodological and gene-regulatory perspectives that bear directly on metastatic mechanisms. First, the use of histochemical marker genes will be reviewed for genetically-tagging tumor cells and following their fate during

metastatic spread in virtually any organ of the experimental animal. Single tumor cells can be followed with relative ease by these approaches. We have now used these marker genes in fibrosarcoma, neuroblastoma, and prostate carcinoma experimental models. Second, we will review evidence that modulation of expression of the CD44 gene is a critical factor in metastatic spread of fibrosarcoma, as well as provide some insight into CD44's mechanism of action. Finally, evidence will be summarized for down-regulation of specific integrin genes by highly-amplified N-*myc* oncogene in the neuroblastoma system. Analyzing these three tumor systems with these and other methodologies will enable us to predict some important advances in metastasis studies during the next decade.

(II) Histochemical Marker Genes to Track Micrometastasis Formation and Subsequent Development into Overt Metastases

(A) Micrometastasis to organs never implicated previously. During early studies of fibrosarcoma and neuroblastoma metastasis in athymic nude mice (14,15), we had been frustrated by our inability to detect the earliest events in primary tumor formation and, more importantly, the earliest events during micrometastasis. Following the precedent set by developmental biologists to study single-cell lineages in embryos (12), we transfected the E. coli *lacZ* gene into fibrosarcoma cells to track these tumor cells. Using the X-gal histochemical staining reaction, single tumor cells could be easily detected in virtually any target organ of the animal (17,18). In later studies (19), we developed the use of Red-gal which stains these cells red, rather than the blue product generated

from X-gal. This approach using histochemical marker genes generated the first studies in any tumor system (17,18). EJ-H-*ras*-transformed Balb/c 3T3 cells were detected undergoing spontaneous metastasis to the lung and liver, as expected from previous low-resolution methods. The sensitivity for detecting tumor cells was so refined that single tumor cells could be detected in sections of lung adherent to the lining of blood vessels, possibly in the act of extravasating; in other cases single tumor cells had already escaped (Fig. 1). Moreover, micrometastases were detectable in the brain and kidney, organs never previously implicated in metastasis of fibrosarcoma tumors (6,17,18). In the case of the brain, these micrometastases were transient and failed to thrive into overt metastases, possibly indicating an environment hostile for fibrosarcoma growth promotion (6).

The versatility of this system was enhanced further by genetically tagging a second fibrosarcoma tumor cell class with the histochemical marker gene, placental alkaline phosphatase (PAP) whose enzyme product remains intercalated in intracellular membranes thereby permitting its enzymatic product to remain intracellular (19,20). The resulting color reflecting PAP enzyme activity could also be manipulated by the chemical nature of the substrate, thereby yielding red, reddish-brown, black, or blue staining cells depending upon which combination of histochemical substrates were used (19). For this system, the PAP gene was transfected into *sis*-transformed 3T3 cells to evaluate their micrometastatic potential from the subcutaneous site (19,21). In contrast to the excellent metastatic potential of the *ras* transformant, virtually no

spontaneous micrometastasis could be detected with the *sis* transformant from the subcutis.

The effectiveness of this approach for evaluating micrometastatic potential in nude mice has been demonstrated for the first time in human prostate carcinoma using PCA cells transfected with *lacZ* (16; C. Miller, J. Holleran, and L. Culp, unpublished data). In the vast majority of animal experimental models of human prostate carcinoma, metastasis can only be observed to the lung and rarely to liver and bone which are high-probability targets of the human disease (22,23). Using the new human PCA cell line, CWR22R (24), generated from a human xenograft in nude mice and transfected with *lacZ*, we have shown that these cells spontaneously metastasize from the subcutaneous site to lung, liver, and brain (J. Holleran, C. Miller and L. Culp, unpublished data). In some of these cases, micrometastases gave rise to well-staining overt metastases. This multiplicity of targets for PCA metastasis should markedly improve our ability to quantitatively and qualitatively evaluate the mechanisms by which difficult-to-study PCA tumor cells undergo metastatic processes.

(B) Micrometastases converted into overt metastases. The ease of detecting *lacZ*-tagged fibrosarcoma cells in micrometastases enabled us to monitor the efficiency with which micrometastases became overt metastases. The lung was the ideal site for such a study since an enormous number of micrometastases could be readily detected in this organ, even with spontaneous metastasis from the subcutis as the site of primary tumor

development (18,19). Many micrometastases persisted in the lung for days and weeks and failed to expand into overt metastases. Others developed slowly into overt metastases while a select few grew very rapidly (within 1 week) into metastases. This phenotypic diversity raises question as to the genetic diversity within the *ras*-transformed 3T3 population responsible for these three subsets of tumor cells. Alternatively, there may be special microenvironments within the lung that permit more successful outgrowth of micrometastases or that may be inhibitory for their outgrowth. These issues require much more detailed molecular biological analyses that histochemically-tagged tumor cells will now permit.

Using the experimental metastasis model provided by tail-vein injections, the ability to detect single fibrosarcoma tumor cells in the lung permitted us to analyze the time course of events for establishment of micrometastases in this target organ (18,25). Within 5 minutes after injection, micrometastases were becoming established in the lung, the quantitation of which revealed maximization in number by 1 hour (Table 1). Within 24 hours, >98% of these foci were "cleared" from the lung while the remaining 1.5% became truly established (18). These results confirm studies from the 1970s of melanoma and other tumor systems for clearance from the lungs of the majority of experimental micrometastases but not all of them (1-4). However, the mechanisms of the selective "clearance" or "resistance" of individual micrometastases remain to be determined in terms of molecular and cellular targeting events.

Lin and Culp (25) took a different approach in addressing micrometastatic mechanisms in the lung. They pretreated their *lacZ*/H-*ras*-transformed 3T3 cells with formaldehyde, ^{60}Co irradiation, or mitomycin C to effect any modulation on micrometastasis size, morphology, or stability. Pre-fixation generated micrometastases that were somewhat larger and more rounded in morphology while the irradiation or mitomycin treatments generated micrometastases that were identical to those of live, untreated cells. However, the irradiated- or mitomycin-treated cells were cleared from the lungs completely and more rapidly than live cells while the fixed cells persisted for much longer periods of time. When untreated cells were mixed with fixed cells and then the mixture injected into the tail vein, all micrometastases were cleared from the lungs, including those containing live cells. These studies indicate the importance of cell surface events (altered by fixation but not by the DNA-directed irradiation or mitomycin treatments) for (a) establishing micrometastases longterm in the lung and (b) guaranteeing their survivability in this organ site (25).

(C) Synergy between two genetic classes during metastasis to the lung. We generated two different oncogene derivatives of Balb/c 3T3 cells--one transformed with EJ-H-*ras* and the other with *sis* (the former is spontaneously-metastatic and the latter nonmetastatic). Tagging these with two different histochemical marker genes (19,21)--the former with *lacZ* and the latter with PAP-- enabled us to evaluate possible synergistic or interferring mechanisms in mixed populations of the two classes. When equal numbers of the two cell

types were mixed and injected into the subcutis (21), primary tumors developed that were regionally concentrated with only one cell type, providing a mosaic of blue-staining or reddish brown staining tumor tissue(16). Sectioning revealed that each region was comprised of only one cell type; there was very little intermixing of the two tumor classes except at the margins of two stained regions. This result for all s.c. primary tumors indicated that clonal dominance of each tumor class must occur to give rise to this regional pattern (16). These studies also indicate that the two oncogenes change the gene expression pattern differently in the same Balb/c 3T3 parent population such that selective growth patterns can be observed in the earliest primary tumors (16).

When mixtures of these two fibrosarcoma classes were injected into tail veins, synergy between the two classes became evident when quantitating sites and qualitatively analyzing cell distributions in the lung (19,21) [Table 1 and Fig. 2]. Most micrometastases were comprised of only the *ras* transformant or the *sis* transformant based on homogeneous staining of blue or red sites (both in whole-organ staining as well as in section staining) (Fig. 2A and B). However, a significant fraction (>7%) of sites that became stably established after 24 hours contained both cell types (Table 1; Fig. 2A and B). When *sis*-transformed cells alone were injected into tail veins, all their micrometastases were cleared from the lungs within a few days, demonstrating the absence of a stabilizing set of conditions for these transformants and contrasting with the stability of *ras*-transformed micrometastases [Table 1]. With mixtures of these two cell classes, the *ras* population provides some unknown stabilizing

influence on the *sis* population such that the latter persist as micrometastases and some develop into overt metastases containing both classes of tumor cells (19,21) (Fig. 2C and D). There is an enrichment of the two-cell-class of metastases indicating the synergy between the two during their outgrowth [Table 1]-- the *ras* transformants must be providing environmental cues to neighboring *sis* transformants for stability and subsequent outgrowth. Whether this synergy is mediated by soluble factors secreted selectively by one of these cell types or by extracellular matrix-mediated events remains to be determined. Having two histochemically-tagged cell types permits us to examine gene regulation events in these earliest mixed-cell micrometastases.

(D) Multiple tumor cells in the earliest micrometastases in lung. Injection of *lacZ*-tagged, *ras*-transformed 3T3 cells into tail veins permitted us to evaluate the number of tumor cells in individual micrometastases (18,25). Sectioning, embedding, and histochemically staining these lungs revealed some surprises (25). Rarely were single cells observed in early micrometastases; the vast majority were comprised of 2-7 cells under conditions where single-cell suspensions were being injected into the tail veins. This indicates that fibrosarcoma cells form micro-aggregates during the first minutes in the animal's circulation or that they aggregate at each site in the smallest blood capillaries of the lung. Cardiac perfusion of fixative during euthanasia also revealed details of the lung's microvessels and demonstrated that micrometastases almost always form in the very smallest capillaries. The same results were observed for *lacZ*-tagged human neuroblastoma cells (26) while

our *lacZ*-tagged prostate carcinoma cells remain to be tested in this paradigm. Could multi-cellularity result from physical entrapment of micro-aggregates in these sites or are these sites preferentially adhesive for tumor cells? Use of histochemically-tagged tumor cells and high-resolution molecular biological approaches (see section V below) should assist better definition of these potential mechanisms.

(E) Angiogenesis during tumor progression. Cardiac perfusion of fixative also permitted us to evaluate the formation of new blood vessels in the vicinity of the developing primary tumor by contrasting red-staining blood vessels with blue-staining tumor cells (26). In the case of *lacZ*-tagged neuroblastoma in the subcutis, staining of the entire subcutaneous site or transverse sections of the primary tumor revealed how quickly angiogenesis is promulgated by tumor cell-secreted factors. New blood vessels were seen developing at the periphery of dense populations of tumor cells within 48-72 hours. Several days post-injection, a few of these micro-vessels had developed into sizable blood vessels. By the time the primary tumor had become palpable, one or more of these larger vessels had become a major vessel feeding the central regions of the tumor.

The rapid induction of new blood vessels by tumor cells indicates that a long period of adaptation in the subcutis by tumor cells to secrete angiogenic factors is unlikely. Rather, the tumor cells may be secreting angiogenic factors (10,11) as cultured populations prior to their injection into the subcutis; alternatively, induction of angiogenesis genes occurs in tumor cells within the

first 24 hours of residence. This system can now be used to assay pro-angiogenic and anti-angiogenic factors in ectopic and orthotopic sites of primary tumor development.

(F) Histochemical markers as indicators of tumor cell genetic instability. Since histochemical marker genes do not provide any selective advantage or disadvantage for tumor cell growth at any site in the experimental animal, their activities can be used quantitatively and qualitatively to monitor the relative genetic instability of tumor cell subsets (6,16). This instability was noted in our very first fibrosarcoma tumor studies (19,21). *LacZ*-transfected, *ras*-transformed 3T3 cells retained excellent stainability in culture for >20 passages and in very large primary tumors grown over a period of weeks in the animal. In contrast, PAP-transfected, *sis*-transformed 3T3 cells began losing stainability within 5-10 passages in culture and virtually all large primary tumors contained regions of non-staining cells (16,19,21).

The same spectrum of stability applied to *lacZ*-transfected human neuroblastoma cells (26). One clone yielded excellent stainability for >15 passages in culture and throughout large primary tumors in the subcutis. A second clone, by contrast, was losing stainability within 5-10 passages and virtually all primary tumors using this clone contained large regions of nonstainable tumor tissue.

Three different *lacZ* transfectants of the human prostate carcinoma cell line, CWR22R, also demonstrate this gradient of genetic instability in expression (16; C. Miller, J. Holleran, and L.Culp, unpublished data). Clone H cells stain

very well for >25 passages in medium without drug selection and produced excellent staining primary tumors after 6-12 weeks in the subcutis of animals. Clone B steadily lost stainability over 10-20 passages in culture without selective media and invariably gave rise to tumors with large regions of nonstainable tissue. Clone D was so unstable that virtually all stainability was lost after 5 passages in culture.

The ease of quantitation of histochemical marker gene expression affords us the opportunity to evaluate the molecular mechanisms of this instability and whether it relates to organ-specific metastasis at all. Several mechanisms for loss of reporter gene expression can be envisioned. First, the marker gene may be deleted from the genome or rearranged in such a way that it has lost integrity. Second, transcriptional down-regulation may occur by induction of a new transcriptional-inhibitory factor (trans-acting) in tumor subpopulations. Third, the marker gene may be transcriptionally down-regulated by a hypermethylation mechanism, which is known to alter regulation of many genes in tumor cells (see section III on this point). Fourth, transcription of the marker gene may be perfectly normal but there may be induction of a factor(s) that inhibits protein enzymatic activity in the cytoplasm of cells. As discussed more fully in section V, there are approaches available now that permit resolution of these various possibilities.

(III) Selection and Counter-selection for CD44 Overexpression During Progression and Metastasis

CD44 is a transmembrane glycoprotein on the surface of many, but not all, cell types in animals (27,28). It harbors several binding domains-- its most external domain binds hyaluronan (HA); a membrane-proximal domain homes lymphocytes; and its cytoplasmic tail binds intracellular cytoskeletal elements. Its pre-mRNA is alternatively spliced into a wide variety of splice products, most of which occur in the membrane-proximal/external portion of the molecule responsible for lymphocyte homing. In contrast to the "standard" isoform (CD44s) which lacks all spliced sequences and is the only identifiable product in lymphoid cells and fibroblasts, its "variant" isoforms (CD44v) frequently do not bind HA, have lost lymphocyte-homing ability, and are observed in cell-type-specific distributions among various epithelial cell types (27,28).

CD44 is a likely gene product with significance for progression and metastasis of some human tumor systems (29). First, many primary tumors of human or experimental animal origin have elevated levels of CD44s or CD44v; in some cases, there are different distributions of CD44v in the tumor population from that of the host tissue (30-33). Second, metastases of tumors express more elevated levels of these CD44 isoforms or a different isoform distribution than that observed in the primary tumor, suggestive of unique functions for the spliced variants (34-37). Third, transfection and overexpression of CD44v (38) induces metastatic potential in normally nonmetastatic carcinoma cells. Transfection/overexpression of CD44s in lymphoma cells (39) also induces

metastatic competence. Finally, CD44s on the surface of lymphoid cells with its ability to bind HA is a critical element in the homing of these cells to adhere to blood vessel endothelial cells and their successful extravasation from vessels (40,41). These processes resemble those undertaken by metastatic tumor cells as they migrate to target organs.

Because of these functional implications for metastasis, we undertook analyses of the significance of CD44s in our fibrosarcoma systems--i.e., Balb/c 3T3 cells transformed with either a *ras* oncogene to make them metastatic or with the *sis* oncogene which does not confer metastatic competence (as described in section II). We also analyzed nontumorigenic revertants (IIIA4 cells) of the *ras* transformants that had lost the transforming *ras* oncogene (14). These studies have revealed a remarkable plasticity of expression of CD44s in these cells during progression and metastasis, as delineated below (42-45).

(A) Oncogene-dependent regulation of mouse CD44s gene expression. FACS (fluorescence-activated cell sorting) analysis of Balb/c 3T3 cells demonstrated that they had modest levels of mouse CD44s (mCD44s) on their surfaces and no detectable CD44v isoforms as expected (42). In contrast, *ras* transformants of these 3T3 cells had very high levels of mCD44s and no CD44v isoforms. *Sis* transformants had levels of mCD44s comparable to those of 3T3 cells while revertant IIIA4 cells had reduced expression levels from the high levels observed in the original *ras*-transformed population. These data indicated correlation of metastatic competence with the elevated levels of mCD44s in the *ras* transformants (42). A second correlation was also made--

the high mCD44s-expressing *ras* transformants could bind exogenously-added HA very well while the *sis* transformants and the revertant cells could bind very little HA in a CD44-dependent manner. When a wide variety of nude mouse primary tumors and lung metastases, derived from the *ras* transformants, were evaluated by FACS and/or Western blotting after transplant back into tissue culture, all demonstrated very high levels of CD44s (42). Therefore, expression of the *ras* oncogene leads to up-regulation in mouse CD44s expression while expression of *sis* is without effect on expression of this gene.

(B) Transfection of human CD44s gene into *sis* transformants--acquisition of metastatic competence and the counter-selection model of progression. We then designed a system to artificially elevate levels of CD44s in transformed cells with a molecule that could be differentiated from the endogenous mouse homolog using specific monoclonal antibodies (43). The human CD44s cDNA gene, under regulation of a very active LTR promoter, was transfected into *sis*-transformed 3T3 cells in an effort to test elevation of cell surface CD44s (either hCD44s or mCD44s). Three independent and stable transfectants were isolated. There was no elevation of mCD44s in any transfectants. In contrast, there were very high levels of hCD44s on these cells. Furthermore, exogeneously-added HA could bind to the hCD44s on the cell surface but not to the mCD44s, providing further functional discrimination between these two classes of molecules (43).

When the hCD44s-overexpressing transfectants were injected into the subcutis of nude mice (43), very aggressive primary tumors formed within 1-2

weeks. Furthermore, these cells were now highly metastatic to the lung, contrasting with the lack of spontaneous metastasis using the original *sis* transformants. When micrometastatic tumor cells from the lung were isolated back into culture, FACS analyses of their CD44s levels revealed the expected levels of mCD44s and very high levels of hCD44s (Fig. 3B and 3D), consistent with the hypothesis that overexpressed levels of hCD44s led directly to acquisition of metastatic competence.

Similarly, primary tumors in this series were isolated into culture and evaluated for their CD44s levels. This led to a very surprising finding (43). All large primary tumors from transfectants had lost expression of hCD44s while retaining the modest levels of mCD44s; this loss was observed in >20 primary tumors examined (Fig. 3A and 3C). These results indicate that overexpression of the human gene may be counter-productive and/or antagonistic for outgrowth of primary tumor in the subcutis. This counter-selection against hCD44s gene expression contrasted with the high-expression levels of this gene product in all lung micrometastatic tumor cells.

This led us to examine a third tumor population. Micrometastases of some hCD44s transfectants in the lung invariably grew into large overt metastases (43). When large metastases were transplanted back into culture and tested by FACS for CD44s levels, they had lost virtually all of their hCD44s while retaining the expected levels of mCD44s. This would indicate that there is not an organ-specific down-regulation of this gene in the two tissue sites. Rather, aggressive outgrowth of tumor, whether it be the primary tumor or

distant metastases, required down-regulation of hCD44s for some inapparent reason, perhaps linked to its HA-binding ability that is defective in mCD44s.

These important studies led us to hypothesize a selection/counter-selection model of tumor progression in this system (43,46). Elevated levels of hCD44s would be critical to get metastatic spread from the subcutis to the lung. Conversely, outgrowth of the primary tumor and any overt metastases required that the gene be turned off in highly selected subpopulations if aggressive cell division was to be successful.

What is the origin of the lung micrometastatic cells with overexpressed hCD44s (43,46)? Two alternative models for the origin of lung metastatic cells could be envisioned. Perhaps these cells migrate from the subcutis to the lung soon after injection and prior to down-regulation of the hCD44s gene. Alternatively, a small subpopulation of hCD44s-overexpressing cells may exist at all times in the primary tumor; it cannot be readily identified by FACS analyses (or by Western blotting for that matter).

As one approach to address these questions, we undertook a second round of tumor analyses (43). In the first case, primary tumor cells that had lost expression of hCD44s were re-injected subcutaneously into a second group of animals. These cells formed excellent primary tumors, all of which failed to express detectable hCD44s. These primary tumors also gave rise to micrometastatic tumor cells in the lungs of these animals. When these micrometastatic tumor cells were explanted into culture and grown out in drug selection medium to eliminate any mouse lung cells, these micrometastatic

tumor populations expressed high levels of hCD44s in all cases. Therefore, metastatic competence correlated again with acquisition of high levels of cell surface hCD44s. In the second case, lung micrometastatic tumor cells from the first round (high hCD44s expressors) were injected into a second group of animals--all s.c. primary tumors had lost hCD44s expression while lung micrometastatic tumor cells retained high levels of this cell surface protein. These experiments demonstrate the remarkable plasticity of hCD44s expression during progression and metastasis with these *sis* transformants of 3T3 cells.

(C) Possible mechanism of modulation of the hCD44s gene. These studies raised the question of how the elevated levels of hCD44s may facilitate metastatic spread while endogenous mCD44s does not convey this competence (46). It appears likely that the ability of the human protein to bind exogenous HA is the critical characteristic that distinguishes hCD44s from the properties of the mouse protein. To prove this, we must test overexpression of a mutant form of hCD44s that is specifically unable to bind HA but is capable of other binding functions. If such a molecule proves incompetent in conveying metastatic competence, then clearly HA binding is a central issue for metastatic spread of fibrosarcoma tumor cells (also will the mutant gene be downregulated in primary tumor cells if it cannot bind HA?). If HA-nonbinding mutants persist in conveying metastatic competence, then there must be some additional and unknown binding activity in this domain of the molecule that is

critical. Further experiments along these lines should prove particularly informative.

Some insight has been provided into the mechanism of modulation of hCD44s expression in these tumor cells (43). Hypermethylation of gene promoter regions has been shown to down-regulate a number of genes in tumor cells (47,48). Testing the hCD44s gene region for sensitivity to digestion with restriction enzymes responding to hypermethylated DNA sequences revealed two very different levels of methylation in or proximal to this gene(43). In the original transfectant cells and lung micrometastatic tumor cells, both of which express considerable hCD44s, there were low levels of methylation in this gene region. In contrast, primary tumor cells and overt metastatic tumor cells that had lost hCD44s protein displayed high levels of DNA methylation in this region. This correlation was further reinforced using aza-deoxycytidine treatment of cells in culture to inhibit DNA methylation (43). This treatment led to high levels of hCD44s in primary tumor cells and only slightly increased the levels in transfectant cells and in lung micrometastatic tumor cells that already had elevated levels. Therefore, it is highly likely that plasticity of expression of this transfected gene in these fibrosarcoma cells is effected by regulation of DNA methylation in this gene's promoter region. To test this hypothesis directly, we can transfect hCD44s gene into these cells under regulation of other active promoters that are much less susceptible to hypermethylation and then test for plasticity of hCD44s expression, aggressiveness of primary tumor development, and acquisition of metastatic competence in this paradigm.

(D) Additional hCD44s expression/plasticity systems. In addition to the *sis* transformants described above, transfection of the hCD44s gene was performed in revertant IIIA4 cells (43). These revertant cells were isolated from a clonal population of K-*ras*-transformed 3T3 cells and were shown to have deleted the *ras* oncogene by some unknown mechanism (14). In doing so, they had lost much of their tumorigenic potential.

Several stable transfectants of IIIA4 cells were isolated after transfecting the hCD44s gene (43). They expressed very high levels of hCD44s protein which was competent for binding exogenous HA. Upon subcutaneous injection into nude mice, they were much more tumorigenic than parental IIIA4 cells. Of particular interest, they had acquired metastatic competence for the lungs of these animals. Lung micrometastatic tumor cells, explanted into culture, expressed high levels of hCD44s while the large primary tumors had lost expression.

This led us to test the third biological system (44). The hCD44s gene was transfected into nontumorigenic Balb/c 3T3 cells to determine if their phenotype was altered in any way. Several stable transfectants were isolated with low, intermediate, or high levels of hCD44s on their surfaces; in all cases, exogenous HA binding was proportional to the amount of cell surface hCD44s and unaffected by mouse CD44s. Of particular note, the highest hCD44s-expressing clone was tumorigenic in the subcutis of nude mice and subpopulations of these cells formed spontaneous micrometastases. In agreement with the other two systems, the primary tumor cells lost expression of

hCD44s while the lung micrometastatic tumor cells retained high levels of hCD44s (44).

This latter 3T3 system was explored further by testing whether these hCD44s levels, observed with tissue cultured populations, could be reflective of tumor populations in vivo (44). Using immunohistochemical approaches, we demonstrated that fixed sections of the primary tumor tissue retained immunohistochemical stainability for mouse CD44s but lacked any significant levels of human CD44s. In contrast, small foci of tumor cells in the lung expressed high levels of hCD44s and low levels of mCD44s (44). These experiments confirmed the results of tissue culture analyses of these various tumor populations and discounted an artifact of altered expression upon tissue culturing of these populations.

(E) Experimental metastasis system to evaluate functional significance of overexpressed hCD44s. The results described above suggest that overexpressed hCD44s facilitated metastatic spread of normally-nonmetastatic *sis* transformants and other cell types to the lung. One approach for testing this hypothesized mechanism involves injection of tumor cells into tail vein blood vessels to evaluate later steps in metastatic spread but not the initiating events. This approach would not be evaluating the intravasation steps into blood vessels at the site of the primary tumor. Since the plasmid used for transfection of the *sis* oncogene into these transformants also harbored the hygromycin B resistance gene, we could quantitate lung colonization by enumerating drug-resistant colonies grown out in culture after harvesting lungs and dispersing

them into single-cell suspensions. This approach yielded some very important mechanistic information for the significance of overexpressed hCD44s in the early events of micrometastatic establishment (45).

Untransfected *sis*-transformed cells generated a low number of micrometastatic foci in lungs at any time point examined after tail vein injection--e.g., 1 hour, 24 hours, and 4 weeks (45) (Fig. 4). In contrast, hCD44s transfectant cells yielded three times that number of foci at 1 hour, more than ten-fold that number at 24 hours, and ten-fold more at 4 weeks (Fig. 4). These results indicate that high hCD44s levels (a) greatly improve initial implantation of micrometastases in the small blood vessels of the lung within the first hour in the circulation and (b) further improve the stabilization of these micrometastases during the clearance mechanisms that operate between 1 and 24 hours. Furthermore, persistence of these micrometastases over the next 4 weeks reveal a lasting effect on their residence in the lungs.

These culture-isolated colonies were also evaluated by FACS for levels of hCD44s and its ability to bind exogenous HA (45). As expected, the lung-colonizing population of transfectant cells had very high levels of hCD44s and excellent ability to bind HA at the 1 and 24 hour time points. In contrast and consistent with plasticity of hCD44s gene expression, the 4 week-harvested cells had lost much of their cell surface hCD44s and their ability to bind HA. These results confirm the importance of cell surface hCD44s in establishing and stabilizing micrometastases but not in promoting their eventual outgrowth in the lung into overt metastases.

The next series of experiments in this paradigm tested the various tumor cell types described in section IIB above and harvested after s.c. injection of hCD44s-overexpressing transfectants (45). hCD44s-negative primary tumor cells, injected into tail veins, gave colony numbers from the lungs that were not statistically different from those of untransfected *sis*-transformed cells. In contrast, hCD44s-overexpressing lung micrometastatic tumor cells, injected into tail veins, gave colony numbers that were 7-10 fold higher than primary tumor cells and comparable to the numbers observed with the original transfectant cells. FACS analyses revealed very high levels of hCD44s on these cells and excellent binding of exogenous HA as well. These results confirm the correlation of high levels of HA-binding hCD44s on cells with their efficiency at colonizing the lung microvasculature (45,46).

In the experimental metastasis assays described above using hCD44s-overexpressed cell classes, a large number of overt metastases grew out within 3-5 weeks (45). Re-isolation of these cells into culture, based on their selection using the drug resistance marker on the transfected plasmid, yielded populations that were depleted of cell surface hCD44s and displayed poor HA binding. These results also confirm those of section IIB and illustrate the selection against high levels of hCD44s when tumor outgrowth becomes aggressive, whether it is the primary tumor in the subcutis or large metastases in the lung (46).

Two general models for these results can be proposed (45,46). First, hCD44s and its ability to bind HA could promote formation of small aggregates

of tumor cells in the circulation that would subsequently be trapped in the microvessels of the lung--i.e., promotion of tumor cell:tumor cell adherence. The second model involves more effective adherence of tumor cells to endothelial cells of these blood vessels by overexpressed hCD44s on the tumor cell surface binding to HA on the surface of the endothelium. Such binding may be relatively weak, as suggested by studies of lymphoid cell homing to sites of inflammation (40,41). Because microvessels do not experience the fluid shear stress that occurs in larger vessels, hCD44s:HA interactions in microvessels may be strong enough to hold tumor cell aggregates to the endothelium. This may explain why tumor cell aggregates are only observed in microvessels of the lung.

To test these alternatives, hCD44s transfectant cells were mixed with untransfected cells in varying ratios. Then the mixtures were injected into tail veins to test the "specific activity" of the overexpressors to colonize lungs in competition with untransfected cells. Hygromycin B was used to select all tumor cell classes in the lung while puromycin (the drug resistance marker on the hCD44s-bearing plasmid) was used to select transfectant cells specifically. Increasing the proportion of untransfected cells had no adverse effect on the much greater efficiency of colonization by the transfected subset. Consistent with this result, the lung tumor populations re-isolated into culture were tested for levels of cell surface hCD44s and were found to be overwhelmingly high expressors. These results would suggest that tumor cell:tumor cell adhesion is not playing a critical role in this mechanism. They are more consistent with the

model of much improved adhesion of transfectant cells to the endothelium, possibly mediated by HA-binding hCD44s (45,46). This would parallel a similar mechanism of lymphoid cell adhesion to the endothelium at sites of inflammation (40,41).

To test this model more directly, we can transfect non-HA-binding mutants of the hCD44s gene into these cells and determine if colonization remains low. We can also test whether other cell surface HA-binding receptors (at overexpressed levels) can facilitate the much improved colonization of these cells and whether HA oligosaccharides, co-injected into the circulation at the same time as tumor cells, can hapten-inhibit colonization.

(IV) N-myc Amplification in Neuroblastoma: Its Regulation of Integrin Receptor Expression

Human neuroblastoma is a tumor arising from the malignant conversion of a neural crest cell in the embryo destined to become an adrenal gland, a melanocyte in the skin, a peripheral neuron, or a facial bone (12,13). It afflicts young children prior to the age of 10 and can arise at many different organ sites (15,49,50). Some neuroblastoma tumor cell lines are neuritogenic in culture and have been studied as models of peripheral neuron differentiation (15).

N-*myc* is a proto-oncogene in the human genome and a member of the *myc* family of proto-oncogenes whose protein products are regulators of transcription of specific genes (51). This proto-oncogene displays a unique correlation with neuroblastoma tumor biology and progression (49,50,51). It becomes highly amplified in aggressive and metastatic neuroblastomas of

stage III or IV, does not display any unique mutations in metastatic tumors, and is not amplified in less-aggressive stage I or II tumors that do not metastasize. In the same tumor populations, the c-*myc* proto-oncogene is unaffected during progression and metastasis, suggesting that N-myc protein is responsible for transcription of genes other than those regulated by c-myc protein and that these N-myc-regulated genes participate in the aggressiveness of this tumor.

Earlier studies had demonstrated that amplification of the N-*myc* oncogene in neuroblastoma cells correlated with down-regulation of the major histocompatability complex class 1 gene, protein kinase C signaling, and the NCAM gene (52-54). Since the class of extracellular matrix receptors, called integrins (55), are critical elements in the altered relationship between tumor cells and their environment (14,15, 55), we sought to more directly test whether metastatic progression of neuroblastoma, mediated by N-*myc* amplification/overexpression, could be based on altered regulation of integrin genes in these tumor populations. Such evidence was obtained.

(A) Integrin expression in naturally-occurring human neuroblastoma cells. Our laboratory undertook analysis of the integrin receptor classes in three human neuroblastoma tumor classes (56,57). Two of these, IMR-32 and LaN1, display amplified N-*myc* oncogene while the third, SK-N-SH, does not. IMR-32 and LaN1 cells do not display significant amounts of any of the common β1 integrin family members. When these cells were injected into the subcutis (an ectopic injection site) or the adrenal gland (an orthotopic injection site for neuroblastoma), all primary tumors conserved this pattern of poor integrin

expression. The rounded, weakly-adherent morphologies of LaN1 and IMR-32 cells in culture were also consistent with poor integrin expression.

In contrast, SK-N-SH cells, harboring a diploid number of N-*myc* genes, expressed significant amounts of $\alpha 2\beta 1$, $\alpha 3\beta 1$, and smaller amounts of $\alpha 1\beta 1$ (56,57). These levels of integrins were conserved in all subcutaneous and adrenal gland primary tumors derived from this cell line. When these populations were analyzed by FACS, approximately one-half of the parental SK-N-SH cells expressed $\alpha 3\beta 1$ on their surfaces while the other half did not. Both s.c. and adrenal primary tumors conserved this dual-expression pattern, indicating that $\alpha 3^+$ or $\alpha 3^-$ cells were not clonally dominant during progression of primary tumors. There was also another distinctive pattern change observed in these studies. The parent SK-N-SH cell line did not express $\alpha v\beta 1$, nor did the adrenal gland primary tumors; in contrast, all s.c. primary tumors expressed $\alpha v\beta 1$, suggesting that upregulation of this integrin may be important in the biology of the primary tumor at this site.

These analyses demonstrate several important findings (56,57). First, integrin expression patterns are generally conserved between the original human tumor population, grown for many passages in culture, and the resultant athymic nude mouse tumors derived from them. Second, this conservation of expression is maintained at both ectopic and orthotopic injection sites. Third, induction of $\alpha v\beta 1$ in the s.c. primary tumors may indicate an important function that is specific to this site, possibly for angiogenesis as suggested from other

αv-expressing tumor populations (26, 58). Finally, clonal heterogeneity for α3β1 expression is conserved in all primary s.c. or adrenal gland tumors, indicating that expression of this integrin is not essential for primary tumor expansion. These studies raise question as to how N-*myc*-amplified tumor cells interact with extracellular matrices, since they lack common fibronectin- and collagen-binding integrins and since these are the more aggressive and metastatic classes of this tumor. The weak adherence of these cells in culture is certainly consistent with this poor integrin expression pattern.

(B) Transfection/overexpression of N-*myc* oncogene in SK-N-SH cells. To test more directly hypotheses relating N-*myc* oncogene overexpression with altered regulation of integrin subunit expression, we transfected an episomal plasmid, pREP4 harboring the human N-*myc* oncogene under regulation of a very active LTR promoter, into SK-N-SH cells which have a diploid number of N-*myc* genes and express a basal level of N-myc protein (59). Using the episomal plasmid for transfection permitted us to raise the concentration of N-*myc* oncogene in these cells by selection with increasing concentrations of hygromycin, the drug-resistance marker on this plasmid (59).

Several notable changes were identified in N-*myc* transfectant cells (59). They displayed an altered morphology in culture from that of well-spread SK-N-SH--they were rounded and easily detachable from the substratum, resembling IMR-32 or LaN1 cells in this regard. Selection with higher concentrations of hygromycin generated a higher precentage of rounded cells and much higher levels of N-myc protein. The levels of N-myc protein in these transfectants was

directly related to the dosage of the N-*myc* oncogene in these cells. Conversely, higher concentrations of hygromycin led to cell populations with greatly decreased amounts of β1 integrin subunit. Plotting the levels of N-myc protein against the levels of β1 integrin subunit in many different transfectants and at many hygromycin selection concentrations demonstrated the inverse relationship between the levels of these two gene products (Fig. 5) (59). Transfection of SK-N-SH cells with an antisense construct of N-*myc* failed to generate these changes. Therefore, these experiments directly relate higher dosages of N-myc protein in neuroblastoma cells to decreasing levels of β1 integrin subunit and are consistent with either direct or indirect regulation of the β1 integrin gene by the N-myc protein.

(C) Two different mechanisms for downregulating integrin expression by N-*myc*. The studies described above left open the issue whether integrin subunits α2 or α3 were also altered in N-*myc* transfectants. This was shown to be the case with the discovery that two different molecular mechanisms are operating (60).

Increasing concentrations of hygromycin, to select for increased levels of N-myc protein, led to cells with decreased levels of both α2 and α3 integrin subunits (60). In contrast, the modest levels of α1 subunit observed in these cells were unaffected by higher N-myc levels, dissociating regulation of this subunit from those of the other two (61). Very little α2β1 or α3β1 were observed

at the cell surface in N-*myc* overexpressors (60) while the levels of cell surface $\alpha 1\beta 1$ was unaffected (61). Evaluation of the levels of mRNAs for these subunits by RNase protection assay showed that levels of $\alpha 2$ and $\alpha 3$ mRNAs were greatly reduced (>85%) in N-*myc* overexpressors while the level of $\beta 1$ mRNA was reduced only 40-50% (Fig. 6) (60). Of significance as well, the levels of *max* mRNA were unaltered in these cells (Fig. 6), demonstrating that this co-effector of N-myc transcriptional regulation was not being co-regulated by the much higher levels of N-myc protein (Fig. 6). Metabolic radiolabeling of proteins in these cells, in concert with pulse-chase analyses, revealed that the half-life of $\beta 1$ was greatly reduced in transfectants while those of the α subunits were unaffected. Therefore, it is likely that the greatly reduced amounts of $\alpha 2$ and $\alpha 3$ subunits in these cells lead to uncomplexed $\beta 1$ which then undergoes degradation (60). The exception to this fate is the modest amount of $\alpha 1$ subunit that does complex with small amounts of $\beta 1$ and successfully gets to the cell surface (61).

Overall, these studies indicate three different regulatory patterns of integrin genes in neuroblastoma tumor cells with highly overexpressed N-*myc* oncogene. First, there appears to be transcriptional down-regulation of the integrin $\alpha 2$ and $\alpha 3$ genes by an N-myc protein-dependent pathway; the *max* co-regulator is unaffected in these cells. Second, downregulation of $\beta 1$ levels are effected at the post-transcriptional level, probably by protein instability caused

by insufficient amounts of α subunit for complexing. Finally, the expression of the α1 integrin gene is unaffected by N-*myc*, consistent with levels of α1β1 that persist in high-metastatic, N-*myc*-amplified tumor populations. It remains to be seen how tumorigenic and how metastatic the N-*myc* transfectants are in nude mice. Do they display the same progression and metastasis patterns as the naturally-occurring N-*myc*-amplified IMR-32 and LaN-1 tumor cells? Both ectopic and the orthotopic (adrenal gland) injection sites must be tested in these regards (56,57).

(D) Is N-*myc* downregulation of integrins tumor-specific? These analyses questioned whether N-myc protein regulation of integrins was specific to neuroblastoma tumors because one or more unknown factors in these tumor cells conveyed tumor specificity. To address this question, we transfected the same overexpressing episomal plasmid of N-*myc* into human Saos-2 osteosarcoma cells, a tumor cell line whose biology and lineage are very different from that of neuroblastoma having been derived from a connective tissue cell type (62).

Several transfectants of Saos-2 were isolated which had greatly elevated levels of N-myc protein (62). While parental Saos-2 cells were well-spread on the substratum and expressed considerable α2β1 and α3β1 integrins, the transfectant cells displayed very little of these integrins on their surfaces and displayed very rounded, easily-detachable morphologies. When mRNA levels and protein turnover patterns were analyzed, the same changes were found to occur in these cells (62) as shown above for N-*myc*-overexpressing

neuroblastomas (59,60). There was transcriptional downregulation of α2 and α3 genes while β1 transcription was minimally altered. In contrast, β1 protein turned over much more rapidly, probably because of lack of a partners and resulting in much lower basal levels of this subunit (62). *Max* mRNA levels were unaltered in transfectant cells, again casting the regulatory role on excess amounts of the N-myc protein. These results indicate that N-*myc* regulatory mechanisms can apply to a different cell and tumor types and are not neural lineage-specific. However, the osteosarcoma system is artificial in that there is no evidence for N-*myc*-dependent regulation or N-*myc* amplification in this tumor under naturally-occurring conditions.

These analyses of N-*myc*-overexpressing neuroblastoma and osteosarcoma cells prompt more extensive study of the interactions (or lack thereof) of N-myc protein, complexed with max protein, with the promoter regions of these three integrin genes. Unfortunately, these promoters have not been well-studied or their cis- or trans-acting sequences deciphered completely (63). If N-myc protein regulates transcription of specific genes, including α2 and α3 integrin genes, in ways that are independent of c-myc protein, then these N-myc mechanisms may be targetable for interfering with metastatic competence of these tumor cells.

(V) Perspectives on "Regulating" the Metastatic Phenotype

The findings described in the preceding sections, when combined with new high-resolution methodologies, enable us to speculate on future directions

in deciphering and possibly interfering with metastasis. First, we will be able to more precisely define the small cell subpopulations in which metastatic competence is being acquired as a consequence of altered regulation of genes, such as CD44 and integrin receptors. Second, by defining which genes are critical for metastatic competence, clinicians will be afforded much more powerful molecular tools for predicting when and where metastasis occurs. A corollary of this is that we should be able to generate reagents that specifically disrupt the functions of these metastasis-conveying gene products while minimally interfering with their "normal" functions in well-behaved cell populations. Finally, if we are able to define organ-specific regulation of some genes in tumor subpopulations, interference with the functions of these genes may permit us to inhibit metastasis specifically to that organ. Some examples of these approaches are offered below. Angiogenesis may be a particularly important target for antagonizing the metastatic process and other chapters in this volume deal with this issue specifically.

(A) Histochemical marker gene-tagged cells for drug screening studies. As shown in section II above, tumor cells tagged with histochemical marker genes can be easily visualized and quantitated in lung and other organs during experimental metastasis. Coinjection of marker gene-tagged tumor cells and candidate metastasis-inhibiting drugs will facilitate identification of useful drugs in several ways. Many drugs can be screened for metastasis-interfering properties using fewer animals and much shorter assay times. In addition, different routes for introducing these drugs into animals can be monitored for

the potential to interfere with spontaneous metastasis after either ectopic or orthotopic injections of tumor cells.

(B) Laser-capture microdissection--a high-resolution tool to analyze gene expression patterns in single tumor cells. The recent development of laser-capture microdissection (LCM) of tissue sections (64-66) permits investigators to select one or a few tumor cells in a tissue environment to analyze. Gene products may be detected in these select cells by immunohistochemistry or by their specific functions (65,66). Expression of any gene may also be analyzed at the transcriptional level by RT-PCR or in situ hybridization (64,66). Furthermore, use of histochemical marker genes will greatly facilitate the identification of tumor cells, to be selected by LCM, in experimental animal models as discussed in section II above. These markers discriminate the tagged tumor cells from untagged neighboring host-organ cells, whose gene expression pattern must also be considered. Therefore, LCM in combination with histochemical tags should be effective at determining whether "normal" host organ cells, adjacent to the primary tumor or metastatic cells, have altered gene regulation programs from their distant counterparts; conversely, the possible altered regulation of tumor cell genes by neighboring host cells can be addressed.

LacZ- or PAP-tagged tumor cells can be detected in fixed sections by first performing histochemical staining and then identifying the appropriate small subset of cells for subsequent laser capture. When two genetically-different tumor classes are tagged with different marker genes as shown in section II, this

approach becomes particularly beneficial in addressing clonal dominance of one cell type over another in specific regions of the primary tumor and in potential metastatic target sites. These dual approaches should prove particularly effective in deciphering the gene expression patterns in micrometastases as they form.

LCM, in combination with histochemical markers, can be used to determine when and where the CD44 gene is being upregulated (or different isoforms expressed) with RT-PCR and primers recognizing specific CD44 splice products. Does this regulation occur early in primary tumor formation or is it occurring in small subpopulations at later stages of the primary tumor? These approaches will permit us to determine whether selected tumor subpopulations, with altered expression of CD44, occur randomly throughout the primary tumor or only in regions adjacent to larger blood vessels, the latter possibly predicting more effective intravsation into blood vessels for eventual metastatic spread.

As reviewed in section IV, neuroblastoma metastasis correlates with N-*myc* amplification and subsequent downregulation of $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrin receptors, but not the $\alpha 1\beta 1$ receptor. Using LCM in combination with marker-tagged neuroblastoma cells, we can test a variety of hypotheses relating the absence of the two integrins to acquisition of metastatic competence. As an example, *lacZ*-tagged/N-*myc*-amplified neuroblastoma cells can be mixed with PAP-tagged/N-*myc*-amplified neuroblastoma in which $\alpha 2$ integrin subunit expression is upregulated by transfection with an $\alpha 2$ gene under control of a

very active promoter. Do both cell types contribute to formation of the primary tumor or does one cell type clonally dominate? By LCM and by following the two histochemical tags, we can determine the relative distributions of the two cell types near major blood vessels in the primary tumor and near other host tissue sites that may be synergistic. Do both cell types metastasize to the same or differing organs? These experiments may reveal some level of synergy between two classes of tumor cells. LCM could be used to examine the gene expression patterns in the earliest micrometastases of these different organs.

(C) Interfering with the functions of genes critical for metastasis. The studies of sections III and IV indicate the relative importance of two different gene classes in the progression of two different tumors in apposing regulation schemes--CD44s in the case of fibrosarcoma metastasis and integrin receptors for neuroblastoma metastasis. In the former, overexpression of the CD44s appears important for conveying metastatic competence while for neuroblastoma downregulation of integrin expression appears important. The identification of such genes raises the issue as to whether antagonists can be identified that inhibit metastasis effectively, based on the structure and function of the metastasis-targeting molecule(s). Hopefully, such an antagonist would not inhibit the normal functions of this molecular class in host organ cells.

An early indication of the possible success in this approach was the demonstration of small synthetic peptides, containing the Arg-Gly-Asp-Ser (RGDS) sequence, to effectively inhibit colonization of the lung when they were co-injected with melanoma and other tumor cells injected into the tail veins of

experimental animals (67,68). This approach proved successful because some integrins on tumor cells recognize an RGDS sequence in target extracellular matrix ligands, such as fibronectin, laminin, and certain collagens. These target ligands in the lungs of the animal would be the "natural" adherence site for tumor cells and provide the stabilization mechanism for eventual extravasation into lung tissue to establish micrometastases. Remarkably, there was very little toxicity to other tissues of the animal when high dosages of these synthetic peptides were injected.

Histochemical marker gene-tagged cells will provide much more facile and quantitative approaches for testing inhibition of metastasis by other haptens or antagonists of specific molecular domains. As examples, specific spliced sequences of CD44v on the surface of some carcinoma cells may provide binding sites for as-yet unidentified receptors on the surface of endothelial cells, thereby facilitating development of micrometastases (27-30). Synthetic peptides may be identified which inhibit this specific binding function while leaving the other generic binding functions of CD44v unaffected; these peptides would be ideal candidates for specific inhibition of metastatic spread of some carcinoma tumor types while being minimally toxic to other cell and tissue types.

CD44s is the simplest member of the CD44 family and has been shown important in metastasis of fibrosarcoma as reviewed in section III via improved colonization of the lung once tumor cells entered the circulation. Some monoclonal antibodies to specific epitopes of CD44s may be identified that interfere with metastatic spread while having limited inhibition of normal

lymphocyte or connective cell functions. Alternatively, the hyaluronan-binding function of CD44s appears very important in the lung colonization function of this molecule. Therefore, small oligosaccharides of HA may hapten-inhibit this colonization process and afford a complementary therapeutic approach with other drug modalities. As much more molecular information becomes available on the multiple binding functions of this complex class of cell surface receptors, we may be able to design dominant-negative inhibitors of one or more of these specific functions and greatly improve the tumor-targeting potential of such antagonists.

Molecular biological approaches may also prove useful for interfering with metastatic processes. An antisense gene for CD44s may inhibit metastatic spread of lymphoma (39) or fibrosarcoma (46) if it could be expressed in a retroviral construct that infects tumor cells more effectively than any neighboring host tissue cells. A similar and more specific approach might be considered for CD44v isoforms-- use of antisense constructs with sequences targeting specifically the alternatively-spliced domains to inhibit carcinoma metastasis (46). In the case of integrin receptors where downregulation occurs commensurate with metastatic spread of neuroblastoma, retroviral infection with an $\alpha 2$ or $\alpha 3$ integrin subunit gene, regulated by a high-activity promoter, may improve extracellular matrix adhesion of the primary tumor population and subsequently inhibit any spreading potential of these cells by other adhesion mechanisms. This is comparable to the overexpression of transfected E-

cadherin gene in some carcinoma cells, thereby inhibiting their invasion and metastatic behavior (69,70).

(D) Will genetic instability in tumor populations help or hinder in targeting the metastatic phenotype? It is fair to say that one characteristic of malignant cells is their ability to generate many genetically-different subpopulations as the primary tumor expands, thereby providing the versatility that permits metastatic spread for highly-selected subpopulations (1-5). The small number of these subpopulations may provide greater efficiency at inhibiting their functions but may require weeks or months of patient treatment to do so because metastatic-competent subpopulations may be generated in the primary tumor on a regular basis. If there were some way to specifically kill such subpopulations by inhibiting their metastatic-competence functions, chances for success in treating the patient may be greatly improved. Greater knowledge of apoptotic and other cell death mechanisms in tumor cells, some of which may be tumor- or cell type-specific, may afford the complementary treatment approach that will ultimately prove successful. In response, however, the versatility in generating many different subpopulations may also lead to antagonist-resistant variants within the primary tumor that can overcome the treatment modality and still metastasize to various target organs. In any case, greater knowledge of the various mechanisms for generating genetic instability in tumor populations will be a prerequisite for more effective targeting of the metastatic phenotype.

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Table I Quantitation of pulmonary micrometastases/nodules[a]

	APSI injected singly			*APSI Co-injected with LZEJ*				
Time of sacrifice	*Micro-metastases*[b]	*Staining nodules*[c]	*Non-staining nodules*[c]	*Double staining foci*[d]	*LZEJ nodules*[c]	*APSI nodules*[c]	*Non-staining nodules*[c]	*Double staining nodules*[c]
1 h	2,500-3,000 (100)	0	0	500 (100)	0	0	0	0
6 h	700 (28-23)	0	0	122 (24)	0	0	0	0
24 h	104 (4.2-3.5)	0	0	24 (5)	0	0	0	0
3 weeks	38 (1.5-1.3)	10	0	3 (0.6)	26	20	7	3
5 weeks	37 (1.5-1.2)	54	17	ND[e]	ND[e]	ND[e]	ND[e]	ND[e]
7 weeks	8 (0.3)	10	5	ND[e]	ND[e]	ND[e]	ND[e]	ND[e]

[a]Mice (24 for two separate experiments) were given i.v. injections of $1x10^5$ APSI cells alone or as a mixture with $1x10^5$ LZEJ cells as indicated. At various times post-injection, mice were sacrificed; whole lungs removed, rinsed with PBS and stained with X-phosphate (or sequentially with X-gal and then with X-phosphate/NBT in the case of co-injections of LZEJ and APSI cells). These values for $1x10^5$ LZEJ cells injected alone have been published previously (18). [b]Values = Number of micrometastases determined with the use of a dissecting microscope. Values in parentheses represent the number of foci remaining in the lung as a per cent of the 1 h value. [c]Denotes nodules of considerable size (>100 cells). Nodules which were heterogeneous in their staining of the histochemical marker genes are referred to as non-staining nodules. [d]These are the number of micrometastases containing both LZEJ and APSI cells in co-localized foci. Values in parentheses represent the number of foci remaining in the lung as a per cent of the 1 h value. The maximal number of foci of all cell classes (LZEJ-only plus APSI-only plus co-localized foci) observed at any time point was 6-7,000. [e]Not determined. [Taken from Lin *et al.* (21) with permission]

Figure Legends

Fig. 1. Tumor cell adherence and extravasation in a lung blood vessel. *Ras*-transformed, *lacZ*-tagged 3T3 cells (17,18) were injected into tail veins of athymic nude mice. Thirty minutes post-injection, an animal was sacrificed and the lung was excised, fixed, and embedded in methyacrylate for cutting into 4 μm thin sections after X-gal staining (6). X-gal-staining tumor cells can be observed after escape from blood vessels in the lung tissue (e.g., small arrow). Blood vessels are detectable as red staining (staining red at broad arrow) with alkaline phosphatase reagent while tumor cells are detectable as small clusters of blue staining within the blood vessel (open arrow). X400.

Fig. 2. Two genetically-different classes of tumor cells establish multiple classes of micrometastases and overt metastases in lung. *Ras*-transformed, *lacZ*-tagged 3T3 cells (LZEJ) were mixed with *sis*-transformed, PAP-tagged 3T3 cells (APSI) after detachment from their respective tissue culture dishes. The equal-number mixture was injected into the tail veins of nude mice and at the specified times animals were sacrificed. Lungs were excised, fixed, and in some cases stained as whole organs or in other cases embedded in methyacrylate and sectioned after dual staining--first for β-galactosidase (red staining using Red-gal) and then for placental alkaline phosphatase activity (blue staining using X-phosphate substrate). Homogeneous micrometastases of LZEJ cells are denoted with small arrows; homogeneous micrometastases of APSI with arrowheads; and micrometastases containing both cell types with broad-open arrow. **[A]** 1 hour after tail vein co-injection. This is a section of

lung in which all three classes of micrometastases could be readily identified, with a sizable fraction containing both cell types. These are quantitated in Table 1. **[B]** 5 hours after co-injection; whole-organ staining. Note that all three classes of micrometastases persist, as detected with whole-organ staining. **[C]** 3 weeks after co-injection; whole-organ staining. Some micrometastases persisted at this late time point (LZEJ cells at small arrow) while others were growing into overt metastases(APSI cells at large arrowhead). **[D]** 3 weeks after co-injection; whole-organ staining. An overt metastasis is shown with both cell types (large-open arrow), demonstrating that these two genetically-related cell types can contribute to the same overt metastasis. LZEJ micrometastases are also evident (small arrow). All magnifications, X220.

Fig. 3. Selection for or against overexpressed hCD44s in tumors from two different transfectants. *Sis*-transformed 3T3 cells were transfected with a cDNA form of hCD44s, regulated by an LTR promoter, on a eucaryotic expression plasmid. Several stable transfectants, expressing high levels of hCD44s were isolated, two of which are analyzed here. Transfectants HUSI-hCD44.5 or HUSI-hCD44.6 were then injected into the subcutis of nude mice. After two weeks, animals were sacrificed and the primary tumor cells grown out in culture, along with lung micrometastatic tumor cells selected in culture based on their drug resistance. These culture populations were then analyzed by FACS for expression of mouse CD44s (using monoclonal antibody KM81) or human CD44s (using monoclonal antibodies GKWA3 or 7.10). **[A]** Subcutaneous tumor after injection of transfectant 5 cells. There has been loss

of expression of hCD44s while mCD44s continues to be expressed at the expected levels. **[B]** Lung micrometastatic cells from the same animal as in [A]. These cells retain high-level expression of hCD44s. **[C]** Subcutaneous tumor after injection of transfectant 6 cells. Again, hCD44s but not mCD44s has been lost from the surface of these cells. **[D]** Lung micrometastatic cells from the animal in [C]. High levels of hCD44s persist in these cells. [Taken from Kogerman et al (43) with permission]

Fig. 4. hCD44s promotes the earliest stages of micrometastasis establishment in the lung. Untransfected or hCD44s-transfectant clone 5 or 6 cells were injected into tail vein blood vessels (experimental metastasis assay with three mice per datum point). At the indicated times, mice were sacrificed, their lungs dispersed into culture, and tumor cell colonies were quantitated in a colony growth assay based on their resistance to hygromycin B killing. Colonies were visualized with Coomassie blue staining **[A]** and enumerated **[B]**. *, $P<0.05$; **, $P<0.005$. [Taken from Kogerman et al (45) with permission]

Fig. 5. Quantitation of relationships between increased hygromycin B selection with N-myc and β1 integrin subunit levels. **[A]** SKMYC2 transfectant cells (59) were grown in the indicated concentrations of hygromycin B and the resultant attached (A) or loosely-adherent (L) populations isolated. N-myc and β1 integrin proteins were quantitated by fluorography and scanning densitiometry. The inset is a log:log plot of the same data showing inverse relationships between the levels of the two proteins. **[B]** Five independent

experiments were quantitated as described in **[A].** [Taken from ref. 59 with permission]

Fig. 6. RNase protection assay for levels of various mRNAs in several neuroblastoma cell lines. SK-N-SH, IMR-32, transfectant SKMYC2, and antisense transfectant SKMYCAS (60) cells were grown in culture and total cellular RNAs prepared. Five micrograms of RNA from each sample were analyzed by RNase protection. In **[A],** probe sequences analyzed levels of α2, α3, and β1 integrins, as well as β-actin as a control. In **[B],** probes included those for max, N-*myc*, and β-actin genes. Migration positions of protected fragments are shown on the right of the panels and their sizes (nucleotides) are shown on the left of these autoradiograms. **[C]** and **[D]** display quantitation of these autoradiograms from several experiments by scanning densitometry. 18s rRNA is included as an internal control for RNA levels. [Taken from ref. 60 with permission]

Fig. 7. Model for regulation of integrin expression by N-*myc* amplification in neuroblastoma tumors. The various levels of regulation of three different integrin subunit genes--α2, α3, and β1--are shown, as well as their decreased amounts when high levels of N-myc protein are observed in both naturally-occurring tumors with N-*myc* amplification, as well as in transfectant populations where N-myc protein is elevated artificially (59, 60). Transcription of integrin subunit genes produces mRNA which is translated into immature proteins. Immature α's and β1 then associate, mature, and translocate to the cell surface.

While β1 mRNA levels are reduced approximately twofold in high-N-myc cells, α2 is reduced to less than 20% and α3 to less than 6% of levels observed in the absence of N-myc protein. While very little (ND) α2 or α3 protein are detectable in these cells, the reduced amount of β1 protein produced is incapable of maturation and appearance at the cell surface, undoubtedly because of its inability to partner with an α subunit. Therefore, only approx. 10% of β1 produced actually shows up at the cell surface, partnered with small amounts of α1 subunit produced in these cells and whose production is unaffected by N-myc protein (61). [Taken from ref. 60 by permission]

Figure 1

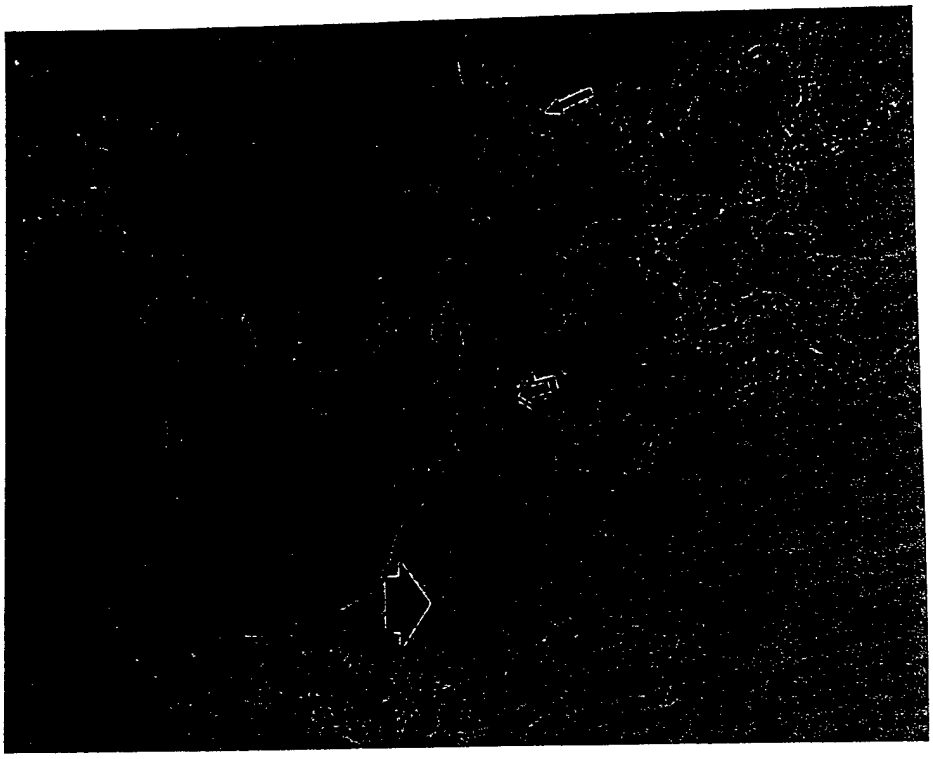
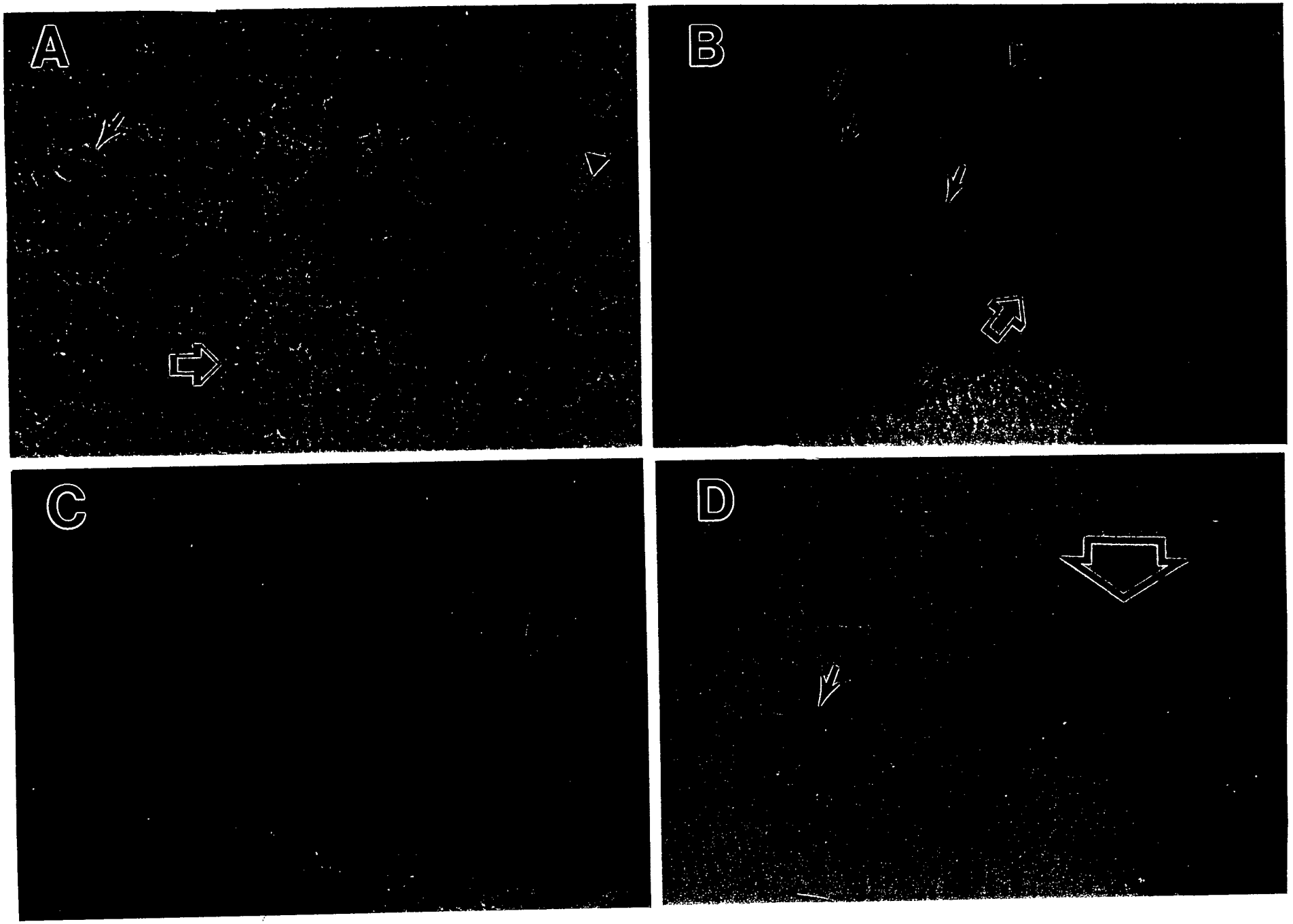
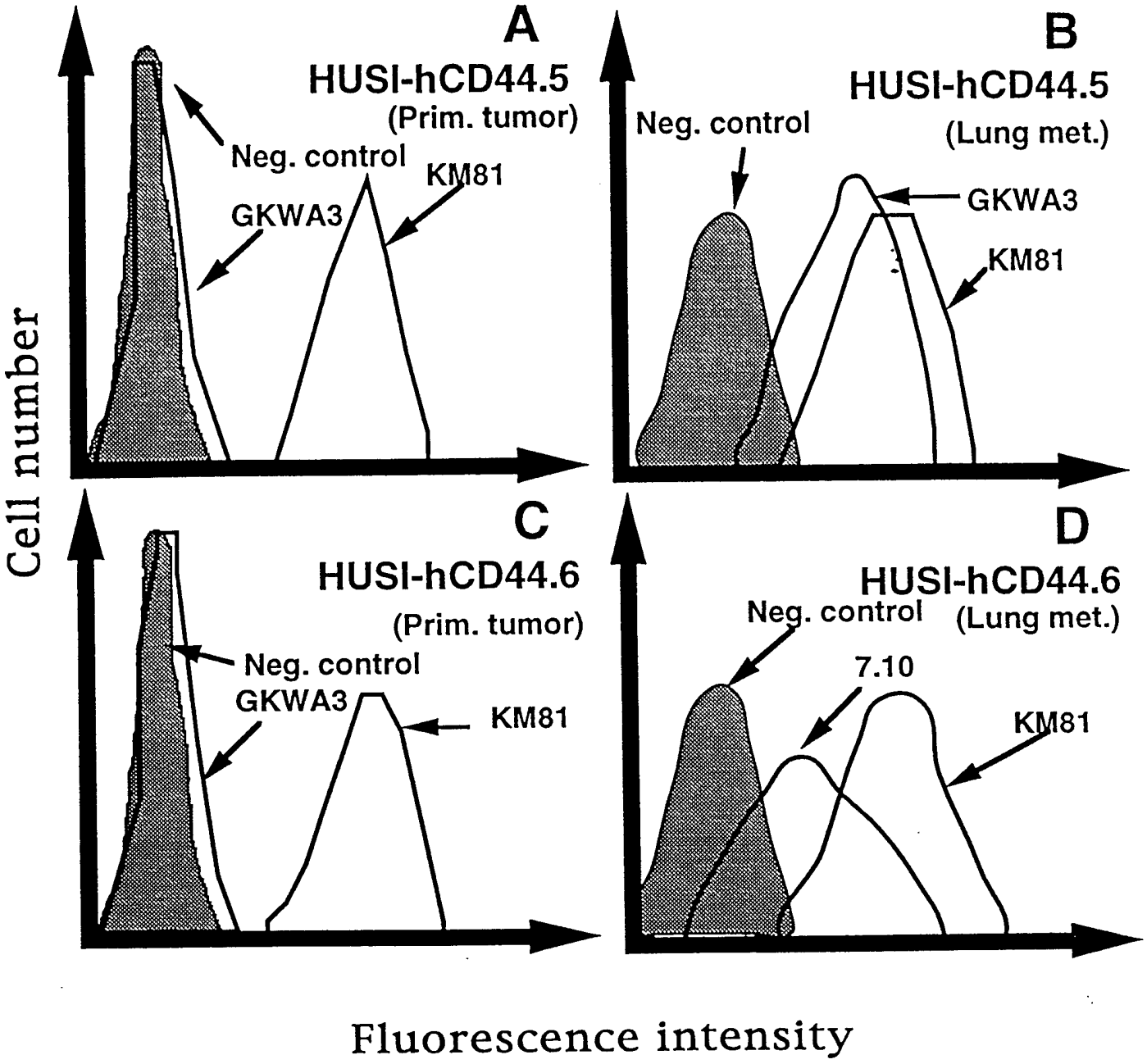


Figure 2




A
HUSI-hCD44.5
(Prim. tumor)
Neg. control
KM81
GKWA3
B
HUSI-hCD44.5
(Lung met.)
Neg. control
GKWA3
KM81
C
HUSI-hCD44.6
(Prim. tumor)
Neg. control
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KM81
D
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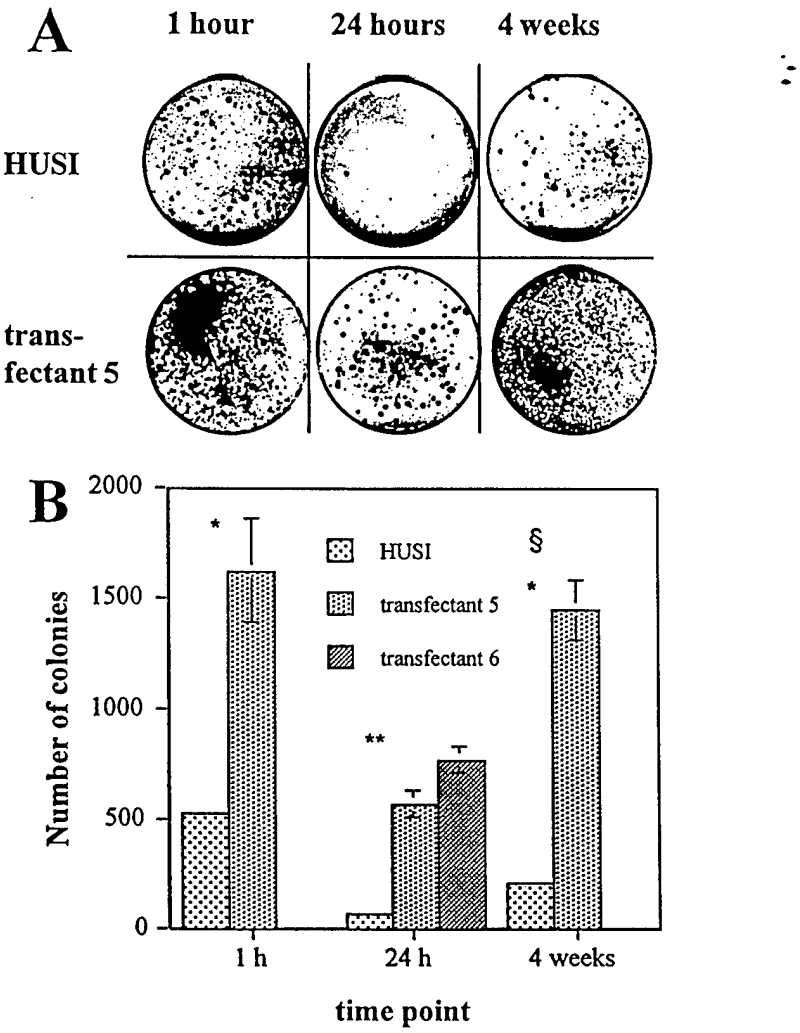
(Lung met.)
Neg. control
7.10
KM81
Cell number
Fluorescence intensity

Figure 3


A
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24 hours
4 weeks
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fectant 5
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2000
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1000
500
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Number of colonies
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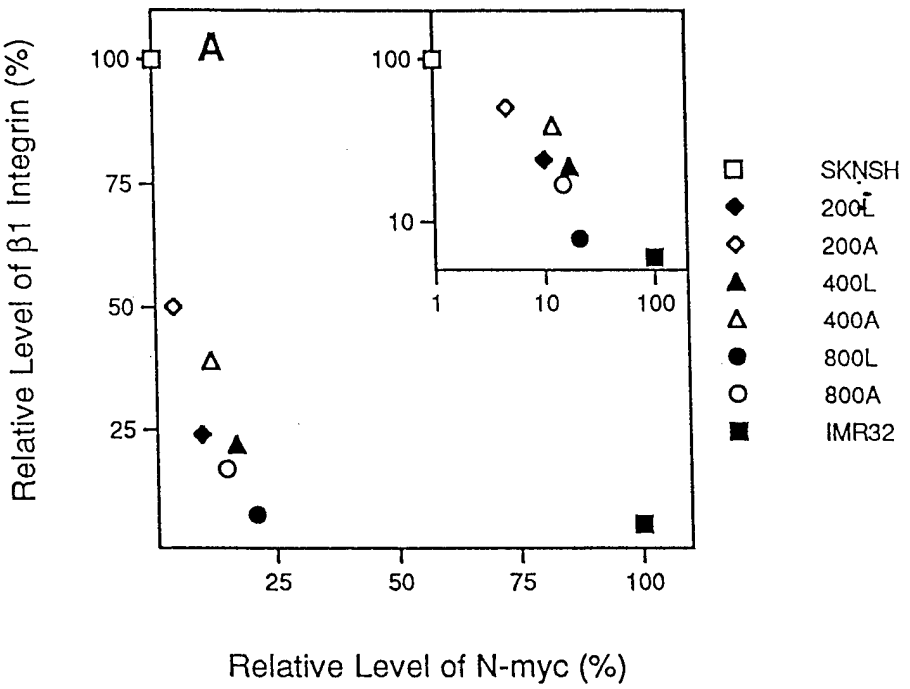
time point

Figure 4

Figure 5.


A
Relative Level of β1 Integrin (%)
Relative Level of N-myc (%)
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200A
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400A
800L
800A
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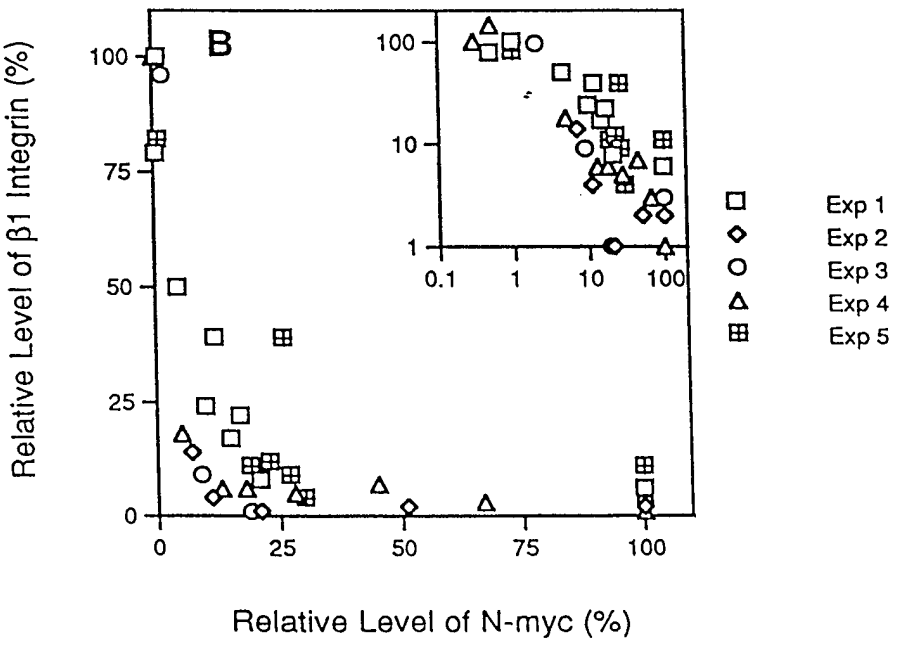
B
Relative Level of β1 Integrin (%)
Relative Level of N-myc (%)
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Figure 6 A and B

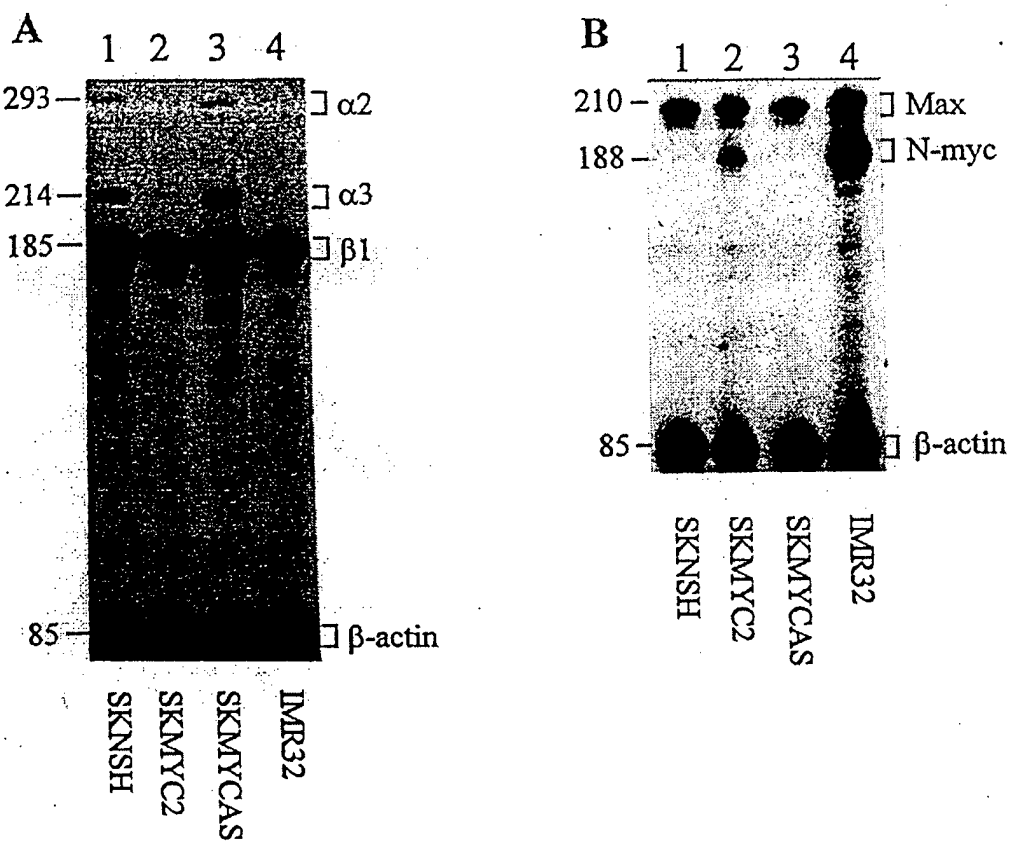


Figure 6 C and D

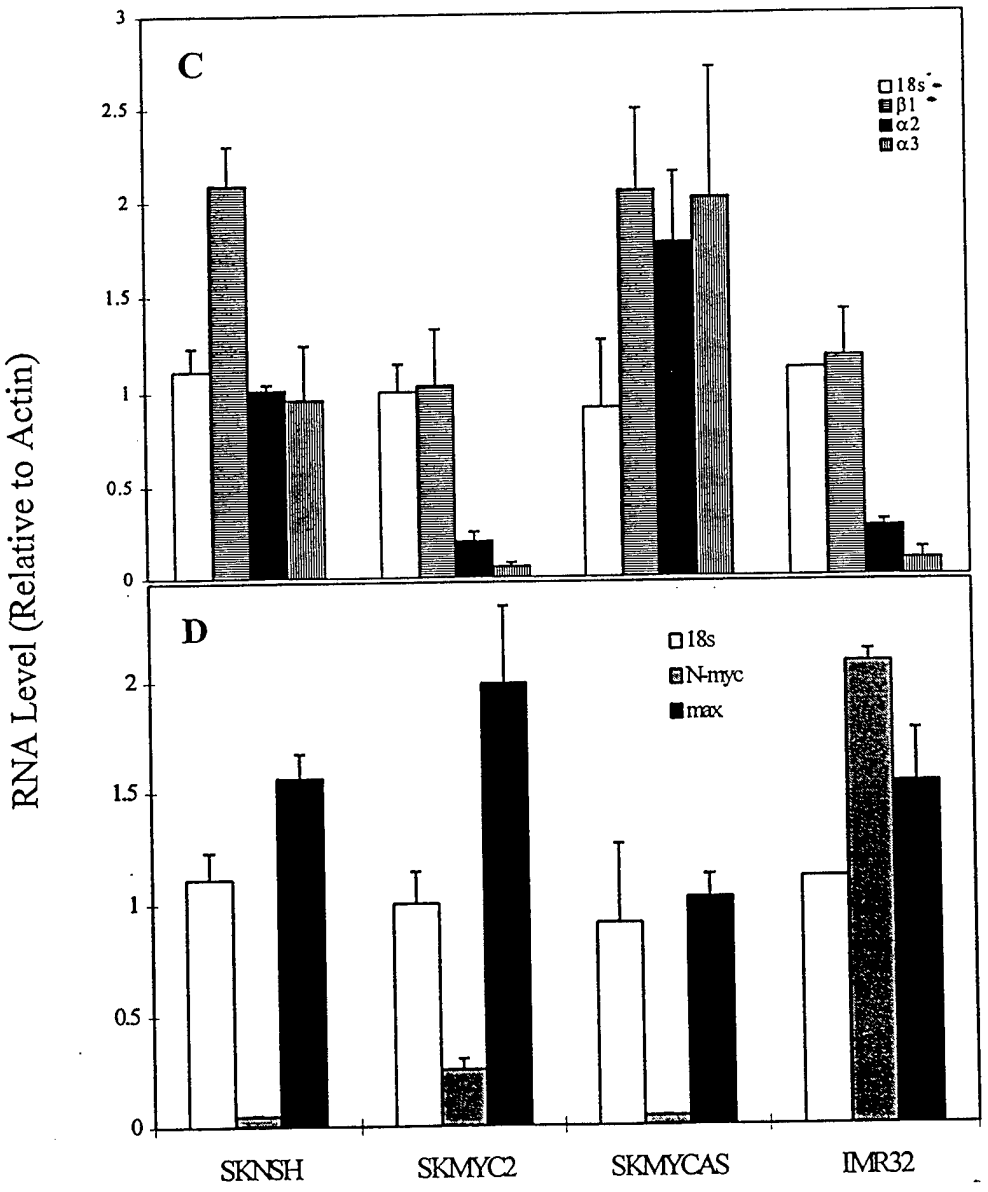
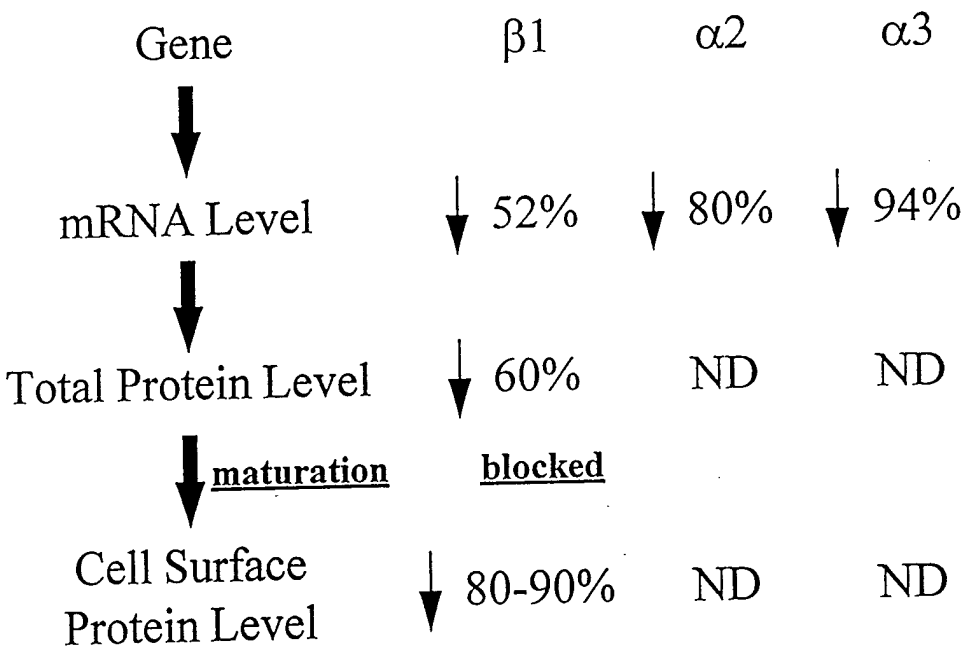


Figure 7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α3
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80%
94%
Total Protein Level
60%
ND
ND
maturation
blocked
Cell Surface Protein Level
80-90%
ND
ND